# ANTIBACTERIAL SUBSTANCES PRODUCED BY MOULDS

## 6. THE PRODUCTION OF CRYSTALLINE PENICIDIN.

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*(Accepted for publication 26th July, 1944.)*

Penicidin is the name given to the substance responsible for the antibacterial activity of a group of Penicillia isolated in South Australia from fruit and vegetables. The occurrence, in the metabolism solutions of these moulds, of an antibacterial substance hitherto undescribed for a Penicillium was first recorded by Atkinson (1943 a)[3] who in two subsequent papers (Atkinson, 1942, 1943 b)[3] described some of the properties and means of identification of crude and partially purified preparations of the substance and called it "penicidin". As a result of further work on methods of production and purification penicidin has now been prepared in crystalline form.

### PRODUCTION OF PENICIDIN BY THE MOULD.

*Variations in Activity of Mould Cultures.*

The original description of penicidin referred to the antibacterial substance produced by the Penicillium, strain 9. Unfortunately the activity of strain 9 gradually disappeared with subcultivation and as a few early attempts to reactivate it were unsuccessful it was set aside and work continued with strains 46 and 67. Both these strains also became inactive after a short time. This variation in activity of the penicidin-producing moulds was a serious obstacle to the progress of the work on the extraction and purification of penicidin. Large batches of metabolism fluid were sometimes inactive, so that further work was held up. Attention was therefore given to means for reactivation and preservation of activity of these strains.

From subcultures on solid medium from some of the oldest stock cultures of strains 9 and 46, spore-suspensions were made and plated on to Sabouraud's agar. Spores from single colonies were picked off into brown sugar medium (Hobby *et al.*, 1942) and some of the resulting liquid cultures were active; from them Sabouraud's slopes were inoculated. These slopes could have been expected to provide an active inoculum for liquid media; variation in activity occurred however in liquid cultures produced from these slopes. Loss of activity was marked in the second and third subcultures. To overcome this difficulty introduced by subculture, spore-suspensions from cultures, which were active in liquid media, were dried *in vacuo* over $P_2O_5$ and served as a source of active inoculum for further large batches of liquid medium for the routine production of penicidin, thus enabling us to continue the work on extraction and purification.

Neither strain 9 nor strain 46, reactivated in this way, has so far produced an activity as great as that which appeared on first isolation. Up to date, strain 67 has not been successfully reactivated.

The temperature of incubation affected the production of penicidin. Metabolism solutions of strain 9 showed maximum activity on the fourth day at 25° C. and on the fifth day at 20° C. At the higher temperature, activity disappeared by the seventh day, whereas at the lower temperature activity did not disappear until the ninth day. As the maximum activity at each temperature was much the same, we preferred the lower temperature on account of the longer duration of activity in the fluid.

A useful rapid method was devised for determining the activity of a solid culture. Instead of inoculating from the culture into liquid medium and subsequently testing for activity, a small block of agar and mould growth was removed aseptically from the mould culture and placed on a nutrient agar plate inoculated with *Bact. typhosum* or *Staph. aureus*. Active cultures produced a zone of inhibition around the agar block on incubation of the plate for 18 hours at 37° C. The

[1] Working with a grant from the University of Adelaide.

[2] Part of the experimental work was done by Mr. N. F. Stanley while a member of a research team working on this problem. Mr. Stanley left the University of Adelaide before the work reported in this paper was completed. His place was taken by Mr. R. A. W. Sheppard.

[3] The first paper written on this subject was submitted to the Medical Journal of Australia in September, 1942, and published in April, 1943. The two subsequent papers (numbers 1 and 2 of the present series) were submitted to the Australian Journal of experimental Biology in November, 1942, and February, 1943, and appeared in this Journal in December 1942, and March, 1943, respectively. Thus the first paper written did not appear until after the two subsequent papers had been published.

plates were prepared in a manner similar to that used in the preparation of plates for assay of penicillin by the ring method (Abraham *et al.*, 1941).

By this agar-block test, Sabouraud's agar appeared to maintain the activity of strain 46 satisfactorily. To determine the reliability of the agar-block test a number of cultures giving a positive result by this test were also inoculated into suitable liquid medium in which they all produced activity. Thus we have a quick and easy test by which we can decide whether a given solid culture is suitably active for inoculation into a large batch of liquid medium for penicidin production.

Being assured of the activity of the solid culture, we investigated the effect of alteration of the type of liquid medium. Czapek-Dox medium with yeast extract (Abraham *et al.*, 1941) was used for the first culture of strain 9 from which crude penicidin was isolated and described (Atkinson, 1942). On this medium or the brown sugar medium of Hobby *et al.* (1942) strains 9 and 46 produced much the same degree of activity. The addition to the brown sugar medium of casein-hydrolysate, which we had found improved penicillin production, accelerated the growth of the mould but did not improve activity, which reached a maximum in the unenriched medium in 4–6 days. In Raulin-Thom medium activity slowly appeared but did not reach in 12 days a strength equal to that attained in 4–6 days in brown sugar medium.

Before strain 9 lost its activity, a single mycelial felt could be used several times to produce active metabolism solution, by drawing off the active culture fluid and replacing with fresh medium, all procedures being carried out aseptically; the replaced fluid achieved its maximum activity earlier than the first fluid. None of the reactivated moulds would produce more than one active fluid from a single mycelial felt.

A more detailed report of this investigation into the control of the activity of the mould will appear later.

*Routine Production of Active Metabolism Solution.*

The results of the previous tests were utilized to modify the method of production of large batches of metabolism fluid containing penicidin. As it was simpler to prepare, brown sugar medium was substituted for the Czapek-Dox yeast extract medium. All cultures were incubated at 20° C. Batches of five litres of medium distributed in bottles in quantities of 250 ml. were inoculated with 1 ml. of a thick suspension of spores washed from a 7-day sporing Sabouraud's agar slope which was active by the agar-block test. The bottles were incubated lying on their sides giving a depth of fluid a little over one centimetre. The mould developed at first as a fine submerged mycelium coming to the surface and covering it in 2–3 days; at the beginning of the fourth day sporing commenced and was usually complete by the fifth day. The onset of sporing generally coincided with the first appearance of activity in the liquid. The felts were a dull olive-green colour with creamy reverse. On the fifth or sixth day the metabolism solution was removed and filtered; it was then ready for treatment by any selected method of purification.

*Antiluminescent Activity of Crude and Partially Purified Penicidin.*

The antibacterial activity of metabolism solutions and partially purified preparations of penicidin was readily detected by inhibition of the growth of *Bact. typhosum*. We found that it could also be detected by the antiluminescence test devised by Rake *et al.* (1942, 1943). Jones *et al.* (1943) used this test for assay of solutions of aspergillic acid (White and Hill, 1943). Antiluminescent activity was manifested by the extinction of the luminescence of a young culture of *Photobacterium fischeri*. Not only aspergillic acid, but other antibacterial substances notably gliotoxin, clavacin and actinomycin A were antiluminescent. Dr. Rake kindly supplied cultures of the *Photobacterium* and tests with penicidin were performed according to his method. Dilutions were made in artificial sea-water medium and to each dilution was added an equal volume of a culture in this medium of *Photobacterium fischeri*, which had been incubated for 24 hours at 16° C. With the dark-adapted eye, readings were made after 30 and 60 minutes. Jones *et al.* (1943) found good correlation between the titre of their routine antibacterial test with *Strep. pyogenes* (read in 18 hours) and the antiluminescence titre (read in 30 minutes) of crude and purified solutions of aspergillic acid. The antiluminescence test was obviously preferable to the antibacterial test on account of the rapidity with which it provided an answer. It was most useful in following the distribution of the active substance during purification procedures.

Crude and partly purified solutions of penicidin were antiluminescent and good correlation was found between the antibacterial titre against *Bact. typhosum* in broth and the antiluminescence titre read after one hour. In determining the antiluminescence titre of metabolism solutions containing penicidin, the lowest dilution tested was 1 in 4; by commencing with such a dilution we eliminated interference in the test by other metabolic products present in small amount, pH value and salt content. Metabolism solutions containing penicillin showed no antiluminescent activity.

Jones *et al.* (1943) found that with aspergillic acid, the antiluminescence test, observed after 18 hours, often showed return of luminescence in dilutions previously extinguished; the aspergillic acid in these tubes was too dilute for antibacterial action and new growth of the *Photobacterium* had occurred. Penicidin behaved differently; in a test with a partly purified solution from strain 67 the antiluminescence titre increased greatly in 18 hours; subcultures (one loopful into 5 ml.

sea-water medium) from the test over the first seven hours showed marked bactericidal action. An antibacterial test in which the penicidin was diluted serially in artificial sea-water medium and each dilution inoculated with *Photobacterium fischeri* gave a much higher titre than that given by Rake's antiluminescence test.

During early work with strains 46 and 67 the reduction of ceric sulphate was correlated to some extent with the antibacterial activity of impure solutions of penicidin and was considered likely to prove a useful quick method of detection and estimation of penicidin. Later work showed the reduction to be due to an impurity accompanying the penicidin through many stages of purification by the first method described below, but possessing no antibacterial activity. No chemical method has so far been found for the detection and estimation of penicidin. The easiest and quickest biological method was the antiluminescence titration which was read in one hour.

## THE EXTRACTION AND PURIFICATION OF PENICIDIN.

Penicidin has been obtained in the crystalline state from the metabolism solutions of three Australian strains of Penicillium, numbers 9, 46 and 94 (described by Atkinson, 1943a; Atkinson *et al.*, 1944).

Before commencing extraction, the metabolism solution was concentrated by evaporation *in vacuo* at a temperature not exceeding 45° C. Providing that the metabolism solution was adjusted to pH 6–7, this procedure resulted in little loss of activity. The solution was generally reduced to about one-twentieth of its original volume.

The first crystalline penicidin was obtained by the following method, although less laborious methods have subsequently been adopted. The concentrated metabolism solution of strain 46 was adjusted to pH 7·5 and continuously extracted with ether for seven hours. This pH value was chosen as earlier work indicated that lower pH values increased the solubility in ether of many inactive coloured substances present in the metabolism solution; at higher pH values penicidin was gradually inactivated. The light brown ethereal solution was evaporated *in vacuo* and the dark-brown oily residue extracted five times with dilute $H_2SO_4$, pH 4; according to earlier tests, at this pH value penicidin dissolved in water but a great deal of the impurity present in the ether extract was insoluble; the impurity was readily dissolved in dilute alkali, pH 8. The dilute acid extracted from the oily residue some impurity which gradually precipitated if the solution was placed at 4° C. A partial purification of the acid solution of penicidin was therefore obtained by standing for several days at 4° C. The precipitate which formed was inactive and the antibacterial titre of the solution did not alter. After separation from the precipitate, the impure penicidin solution was adjusted to pH 7·5 and shaken thoroughly with six separate lots of ether which removed the bulk of the penicidin. The pooled ether extracts were evaporated *in vacuo* and the oily mass treated as before with dilute $H_2SO_4$, pH 4·0. The acid solution was concentrated *in vacuo* to small volume, and on placing at 4° C. crystals of penicidin rapidly appeared. They were separated and recrystallized readily from a very small volume of water.

In a later method of extraction, the concentrated metabolism solution of strain 46 was shaken thoroughly with one-third its volume of ethyl acetate; six extractions removed most of the penicidin, which passed more readily into ethyl acetate than into ether. The ethyl acetate solution was evaporated *in vacuo*, and the yellowish-brown oily residue produced some small crystals on standing at 4° C. The whole residue was treated with several small amounts of warm chloroform, which dissolved much of the penicidin and left behind a great deal of the inactive material. The chloroform solution was filtered and evaporated *in vacuo* to small volume; on placing at 4° C. impure crystals of penicidin appeared. These were separated and dissolved in a little hot chloroform from which the penicidin recrystallized on evaporation. This method resembled that used by Chain *et al.* (1942).

A method resembling that described by Birkinshaw *et al.* (1943) was adopted for the routine extraction of penicidin. By this method crystals were obtained from the metabolism solutions of strains 9 and 94. The concentrated solution was thoroughly shaken six times with one-third its volume of ethyl acetate; the ethyl acetate extracts were pooled, dried with anhydrous sodium sulphate and evaporated *in vacuo*; the residue, which was a yellowish oil, was extracted six times with small volumes of ether. The pooled ethereal solutions were passed through a column of alumina which had previously been treated with dilute HCl and dried at 100° C. The column was washed with ether. Some inactive impurities remained adsorbed on the alumina forming a diffuse brown band high up in the column. The ether filtrate and washings were pooled, evaporated *in vacuo* to a small volume and placed at 4° C. Crystals of penicidin appeared; these were separated, washed in a little ether and dried. Earlier work on chromatographic methods for purification of penicidin showed that alumina which had been well-washed in water and heated at a temperature higher than 100° C. did not effect a satisfactory purification. Ethereal solutions passed through such a column failed to crystallize on evaporation to small volume, and yielded a pale yellow waxy material containing the penicidin.

The yield of penicidin from strains 9, 46 and 94 was small, about 100 mg. or slightly less being obtained from one litre of metabolism solution.

Detailed work on the chemical and biological properties of crystalline penicidin is progressing and will form the subject of a later paper.

## DISCUSSION.

Variation in the production of the antibacterial substance by a mould is not unknown. Waksman and Schatz (1943), studying the production of clavacin by *Aspergillus clavatus* concluded that a single species of a fungus may contain many strains that vary greatly in their capacity to produce antibacterial substances. Jones *et al.* (1943) in studies on aspergillic acid produced by *Aspergillus flavus* recorded enormous variation in the activity of different strains. Degeneration of penicillin-producing ability of strains of *Penicillium notatum* has also been recorded (Foster *et al.*, 1943). Lyophilized cultures of the most active strain are therefore recommended for routine production of the antibacterial substance.

A useful rapid method, the "agar-block" test was devised to test activity of solid cultures. Activity in liquid medium was affected by temperature of incubation, and composition of the medium. The antibacterial activity reached its peak and disappeared more rapidly at 25° C. than at 20° C. The simple brown sugar medium of Hobby *et al.* (1942) was satisfactory for penicidin production and no improvement occurred on addition of nitrogenous enrichments such as casein-hydrolysate.

The final yield of crystalline penicidin, using the best methods so far discovered was disappointingly small owing, we think, to the low activity of the various Penicillia after reactivation.

Penicidin was actively antiluminescent; it shared this characteristic with other antibacterial substances produced by moulds, notably aspergillic acid. Rake *et al.* (1943) noted that among antibacterial substances of microbiological origin, pyocyanase, pyocyanin, streptothricin, actinomycin, gliotoxin, clavacin and fumigacin were antiluminescent while penicillin, gramicidin, gramidinic acid, tyrothricin and flavatin were inactive.

Jones *et al.* (1943) found the antiluminescence titration satisfactory for assaying solutions of aspergillic acid; we have found it useful in estimating the concentration of penicidin in crude and partially purified preparations. So far biological methods only have been successful in detecting and estimating penicidin, and of the available methods the antiluminescence titration provides results in the shortest time.

## SUMMARY.

The production of penicidin by certain Australian strains of Penicillium was investigated.

The activity of strains was readily lost but was restored in some cases by various cultural procedures.

Penicidin was actively antiluminescent. The antiluminescence titration was used for estimation of the activity of crude and partially purified preparations.

The isolation of crystalline penicidin is described.

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# ANTIBACTERIAL SUBSTANCES PRODUCED BY MOULDS

## 7. THE ACTIVITY OF A FURTHER GROUP OF AUSTRALIAN STRAINS OF PENICILLIUM AND ASPERGILLUS.

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*(Accepted for publication 4th August, 1944.)*

In two earlier papers Atkinson (1943 a and b), reported the antibacterial activity of a group of locally isolated Penicillia. The active members, all of which inhibited *Staph. aureus*, were divided into two groups according to their activity against *Bact. typhosum* which was inhibited by Group 2 but not by Group 1. The moulds of Group 1 produced penicillin and those of Group 2 produced penicidin (Atkinson, 1942). The present work records the results of the investigation of a further group of Australian Penicillia and Aspergilli.

### METHODS.

From a culture on Sabouraud's agar a spore suspension was prepared and inoculated into Czapek-Dox medium modified according to Abraham *et al.* (1941) or into the brown sugar medium of Hobby *et al.* (1942). If a culture appeared mixed it was plated and the liquid medium inoculated from cultures from single colonies. In some cases other liquid media were also used. Activity in the metabolism solution was detected by either method, (a) the agar cup test previously described, or by method (b) the ring test described by Abraham *et al.* (1941) for assay of penicillin solutions; two separate ring tests, one with *Staph. aureus* and one with *Bact. typhosum*, were made on each metabolism solution. All metabolism solutions were tested on the fourth day of incubation and many were tested regularly for several weeks. All those cultures proving inactive on the fourth day were tested on at least two later occasions, usually the sixth or seventh day and the tenth or twelfth day.

Another method (c), not involving metabolism solution, was sometimes used. For method (c) the mould was inoculated on to one side of a nutrient agar plate; after several day's incubation two narrow strips of agar each containing a small portion of the mould growth, were cut aseptically from the culture daily until the agar was all used up. The agar strips were placed immediately in a sterile petri dish and inoculated, from the mould growth to the end of the strip, with a streak of *Staph. aureus* or *Bact. typhosum*, and incubated at 37° C. for 24 hours. The distance between the mould growth at one end of the strip and the commencement of bacterial growth along the strip indicated the degree of activity of the mould.

All mould cultures were incubated at 18–20° C.

### RESULTS.

Fifty strains of Penicillium and two strains of Aspergillus comprised the present group of Australian moulds of which 15 Penicillia and both the Aspergilli were active. Table 7 gives the details of the strains and their activities. The numbers given to these strains continue on from those given to the previous group of moulds (see Table 1, Atkinson, 1943a).

When the active Penicillia were grouped as before according to their activity against *Bact. typhosum*, six strains fell into Group 1 and eight strains into Group 2; strain 68 was active only against *Strep. viridans*.

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[1] Working with a grant from the University of Adelaide.

[2] Part of the early experimental work was done by Mr. N. F. Stanley while a member of a research team working on this problem; Mr. Stanley left the University of Adelaide before this paper was completed. His place was taken by Mr. R. A. W. Sheppard.

TABLE 1.

*Moulds showing antibacterial activity after four or more days at 18–20° C.*

| Mould. Number. | Type. | Source. | Group. | Test method. | Activity against. *Staph. aureus.* | *Bact. typhosum.* |
|---|---|---|---|---|---|---|
| 62 | Penicillium | Broad beans | 2 | a | ± | + |
| 67 | Penicillium †terrestre | Plate contaminant | 2 | a | + | + |
| 68 | Penicillium | Plate contaminant | † | a | — | — |
| 76 | Penicillium | Contaminant in blood | 2 | a | + | + |
| 80 | Penicillium | Wood | 2 | a | + | + |
| 87 | Penicillium | 50 years' old wedding cake | 1 | b | + | — |
| 88 | Penicillium | Northern Territory † contaminant of bacteriological culture | 1 | b | + | — |
| 89 | Penicillium | Milk | 1 | b | + | — |
| 90 | Penicillium | South Australian pine wood | 1 | b | + | — |
| 91 | Penicillium | South Australian pine wood | 1 | b | + | — |
| 94 | Penicillium | Paw-paw | 2 | b | + | + |
| P2 | Penicillium (grass-green) | Contaminant, Waite Institute | 2 | b | ± | ± |
| | | | | c | + | + |
| P3 | Penicillium | Contaminant, Waite Institute | 1 | b | + | — |
| | | | | c | + | — |
| P5 | Penicillium | Contaminant, Waite Institute | 2 | b | + | ± |
| | | | | c | + | + |
| P6 | Penicillium | Contaminant, Waite Institute | 2 | b | + | ± |
| | | | | c | + | + |
| A1 | Aspergillus | Contaminant, Waite Institute | – | b | + | ± |
| | | | | c | + | + |
| A2 | Aspergillus | Contaminant, Waite Institute | – | b | + | ± |
| | | | | c | + | + |

When a culture proved to be inactive after four days' incubation it usually proved inactive at all later tests. The activity of the members of Group 1 generally increased up to the eighth day by which time the activity of many of the members of Group 2 had disappeared.

*The Type of Active Substance Produced.*

The metabolism solutions from some of the active moulds were tested for the type of active substance produced. The tests were selected from a number previously described (Atkinson, 1943b). Strains 87 and 88 (Group 1) produced an active substance which behaved at various pH values and extracted into ether in exactly the same way as the penicillin produced by Fleming's strain of *P. notatum* (N.C.T.C. 4222); the metabolism solution of strain 87 gave no characteristic reaction with $FeCl_3$, $KMnO_4$ or HCl. We considered that the activity of these moulds was due to penicillin. Metabolism solutions from strains 67, 76 and 94 gave no characteristic reaction with $FeCl_3$ and from their behaviour at different pH values and the method of extraction of the active substance, were considered to contain penicidin.

*Activity in Other Media.*

Some of the active moulds described in this paper and in the previous paper (Atkinson, 1943a) were grown under the conditions described by Hetherington and Raistrick (1931) for the production of citrinin; some were also cultivated in Raulin-Thom medium. Strains 22 and 23 produced no citrinin and were inactive under the conditions for citrinin production; they were also inactive in Raulin-Thom medium, but each produced on Czapek-Dox medium an amount of penicillin comparable with that produced by Fleming's *P. notatum*. Enrichment of the medium with casein-hydrolysate increased the yield of penicillin with all these strains. Corn-steep liquor, though produced in Australia, was not available for our use; tests with our local strains of penicillin moulds on media enriched with corn-steep liquor would have been very interesting, as this enrichment is recommended by the American workers (Woodruff, personal communication, 1943).

Penicidin production by strains 9, 46 and 67 was not improved by addition of casein-hydrolysate to the medium. In Raulin-Thom medium strain 67 was slightly active in twelve days and strain 9 was active in eight days; the activity was not so marked as that produced in

brown sugar medium in six days, nor was the maximum activity as great. The penicidin moulds numbers 9, 46 and 67 tended to lose their activity on sub-cultivation; but strain 94 has so far remained active.

## DISCUSSION.

Among Australian strains of Penicillium, antibacterial activity is by no means rare, but so far only two types of activity have appeared and are considered to be due to the production of either penicillin or penicidin. An almost equal number of strains of each type has been isolated. No citrinin-producing Penicillia have been found in spite of the use of special methods, in some cases, to encourage the production of citrinin.

The power to produce penicillin appears to extend to many Penicillia and is not a particular characteristic of *P. notatum*. We have had no difficulty in picking up locally numerous penicillin-producing strains of Penicillia. McKee *et al.* (1944) have obtained a substance, flavacidin, probably identical with penicillin, from the growth of *Aspergillus flavus*.

We sent our Penicillia, strains 1 and 22 (Atkinson, 1943a), which are representative Australian penicillin-producers, to the United States for typing. They belonged to the *notatum-chrysogenum* group and closely resembled *P. meleagrinum* Biourge[3]. We sent also our penicidin-producing strains 9, 46 and 67 (see this paper and Atkinson, 1943a). These strains are proving difficult to identify; on first cultivation they resembled *P. terrestre* Jensen but later transfers appeared more fasciculate[3]. One of the other active Penicillia, strain P2 not so far typed, produced a mycelial felt with a marked and distinctive grass-green colour.

In earlier papers Atkinson (1943 a and b) recorded the natural occurrence of penicillin moulds mainly as contaminants of bacteriological culture media, and of penicidin moulds on fruit and vegetables. In the present series penicillin moulds have been obtained from South Australian pine wood and other sources besides the laboratory; penicidin moulds again occurred on fruit and vegetables and probably those labelled "contaminant, Waite Institute" originated from fruit.

The Aspergilli were probably *fumigatus* for which active substances have been described (Waksman *et al.* 1943; Chain *et al.* 1943); their activity is therefore not unexpected.

An interesting comparison may be made between our results and those of Wilkins and Harris (1942, 1943) working with typed series. They found greatest activity among the Aspergilli and the Penicillia, but particularly pointed out that different strains of the same species might vary in activity. Their strain of *P. meleagrinum* Biourge was active against both *Staph. aureus* and *Bact. coli* while our strains resembling this species were active only against *Staph. aureus;* their active strain of *P. terrestre* Jensen inhibited only *Staph. aureus* and they also recorded four inactive strains. Again there is a difference between these results and those which we obtained with our strains resembling this species. Wilkins and Harris (1942, 1943) give no details of the cultural conditions or methods of testing, all of which differed from time to time. It has previously been noted (Atkinson, 1943a), and must be stressed again, that the conditions under which a given fungus is tested markedly affect the result. Our results apply therefore only to the specified conditions of testing.

## SUMMARY.

The antibacterial activity of fifty Australian strains of Penicillium and two Australian strains of Aspergillus has been investigated. Under the conditions of the test fifteen Penicillia and both Aspergilli showed antibacterial activity.

[3] We thank Dr. K. B. Raper of Northern Regional Research Laboratory, Peoria, for providing this information.

Either penicillin or penicidin was demonstrated in metabolism solutions from strains of Penicillium submitted to test.

All active strains were derived from naturally-occurring moulds from a large variety of sources.

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# THE SEROLOGY OF THE TOBACCO MOSAIC VIRUS

## 2. THE ACTION OF VARIOUS SALTS AND SALT CONCENTRATIONS IN THE AGGLUTINATION REACTION.

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*(Accepted for publication 19th August, 1944.)*

In a previous paper Atkinson (1939) described the behaviour of mixtures of tobacco mosaic virus and homologous antiserum under the usual conditions for agglutination tests, namely in physiological saline (sodium chloride, approx. M/7) at 50° C. The system showed several peculiar features, notably the dependence of the antibody on the concentration of serum and the absence of optimal agglutination in the constant-antigen test with dialysed antiserum.

For obvious reasons sodium chloride at normal serum concentration has been accepted as the standard electrolyte for serological tests. This practice takes no account of the nature of the antigen, the natural environment of which may be quite different from a sodium chloride solution, M/7. An example of such an antigen is the tobacco mosaic virus, the natural environment of which contains not sodium, but predominantly potassium often in combination with an organic acid.

Most workers who have attacked the problem of the action of salt in agglutination by antiserum, have confined their attention to sodium chloride, dealing mainly with the effects of altering its concentration. Duncan (1934, 1937) and Platt (1938, 1941) investigated the action of various concentrations of sodium chloride in the agglutination of bacteria by their antisera. The results differed in different systems. Duncan, working with *Bact. flexneri*, found that the antibody optimum depended upon the concentration of sodium chloride used in the test; on the other hand, in Platt's system, consisting of a freshly-isolated, smooth strain of *Br. abortus* and the homologous antiserum from an infected cow, the antigen and antibody optima remained constant in concentrations of sodium chloride from 2M/7 to M/56. In both systems the end-titre of the antiserum depended to some extent upon the concentration of sodium chloride in the test.

Unusual serological reactions probably indicate that the chosen salt or salt concentration is unsuitable. Platt found that his strain of *Br. abortus*, tested one year after isolation, no longer gave uniform serological results but could be induced to do so by "correction" of the concentration of sodium chloride for each test. He suggested that variation in the serological optima with variation in salt concentration indicated an S→SR mutation of *Br. abortus* (that is an increase in salt-sensitivity).

The present work was undertaken in an endeavour to find the most suitable salt solution for serological work with the tobacco mosaic virus. Tests were performed in various concentrations of sodium chloride and a variety of other salts and solutions, some of which were chosen to resemble to some extent the natural environment of the virus. Many of the salts bore little relation to those of serum. When the antigen and antibody each originate from totally different sources, as they do in the tobacco mosaic virus system, difficulty must always be experienced in deciding upon the most suitable diluent.

## EXPERIMENTAL METHODS.

*Antigens.* The preparations of crude and purified virus were similar to those used in the previous work and were supplied by Mr. R. J. Best of the Waite Agricultural Research Institute.

*Antisera.* The antiserum SA, prepared against purified virus and described in the previous paper was used.

*Technique of optimal proportions tests.* The method described in the previous paper was used. For tests involving various salt concentrations, the salt-free reagent (antigen or antibody) was diluted with an equal volume of double-strength salt; this solution contained the reagent diluted 1 in 2 in the required salt concentration and in which subsequent dilutions were made. The tests were observed in an illuminated water-bath of the type designed by Platt (1941), and regulated to 50° C.

## RESULTS.

### *Serological Tests with Sodium Chloride.*

In constant-antibody tests the salt concentration was carefully controlled; the antiserum and solutions of crude virus were well dialysed. In a preliminary test with purified virus (5 mg. per ml.) and antiserum 1/1,200 the O.R. was 15: 1 in M/20 sodium chloride and 2: 1 in M/200 sodium chloride. In the more dilute salt the optimum was particularly clear.

A series of tests was made with crude virus (approximately 2 mg. per ml.) and varying salt and serum concentrations; Table 1 contains the results.

TABLE 1.

*Constant-antibody optima in various sodium chloride concentrations.*

| Serum dilution. | Optimal ratios at sodium chloride concentrations of | | | | | | |
|---|---|---|---|---|---|---|---|
| | M/7. | M/14. | M/28. | M/56. | M/112. | M/224. | M/448. |
| 1/1,000 | 67: 1 | 67: 1 | 33: 1 | 17: 1 | 8: 1 | 4: 1 | 3: 1 |
| 1/2,000 | 67: 1 | 50: 1 | 33: 1 | 17: 1 | 8: 1 | 8: 1 | 4: 1 |
| 1/4,000 | 50: 1 | 33: 1 | 17: 1 | 17: 1 | 17: 1 | 8: 1 | 4: 1 |
| 1/8,000 | 50: 1 | 33: 1 | 33: 1 | 17: 1 | 17: 1 | 8: 1 | — |

Concentration of virus approximately 2 mg. per ml.

The O.R. fell off considerably with fall in concentration of sodium chloride; the prozone increased.

Some serum-virus mixtures were made in serial dilutions of sodium chloride to observe salt optima. The results appear in Table 2.

TABLE 2.

*Virus-antiserum mixtures in various concentrations of sodium chloride.*

| Dilution of crude virus. | Serum dilution. | Ratio. | Sodium chloride concentrations | | | | |
|---|---|---|---|---|---|---|---|
| | | | M/14. | M/28. | M/56. | M/112. | M/224. |
| 1/10 | 1/2,000 | 200: 1 | * | + | — | — | — |
| 1/80 | 1/8,000 | 100: 1 | * | + | — | — | — |
| 1/60 | 1/2,000 | 33: 1 | * | + | + | + | — |

* = first tube to flocculate. + = flocculation in 18 hours.

As all mixtures flocculated most readily in the strongest solution of sodium chloride tested, namely M/14, the sodium chloride optimum lay at or above that concentration. With decreasing concentration of sodium chloride the amount of antiserum required for agglutination increased.

Accurate tests on the effect on the antigen optimum of alteration of salt concentration were prohibited by the impossibility of reading an optimum with dialysed serum. Serum end-titres were determined with undialysed serum and varying concentrations of sodium chloride by commencing with a dilution of serum in water, sufficiently high to reduce the serum salts to approximately the salt concentration required, and subsequently diluting in the required concentration of sodium chloride. For each concentration a range of antigen dilutions was tested; the results are given in Table 3.

TABLE 3.

*Serum end-titres in various sodium chloride concentrations.*

| Antigen dilution. | End-titres at sodium chloride concentrations of M/50. | M/100. | M/200 | M/400. | M/800. |
|---|---|---|---|---|---|
| 1/50 | 1/2,000 | 1/1,000 | 1/500 | > 1/500 | > 1/100 |
| 1/100 | 1/2,000 | 1/2,000 | 1/500 | > 1/500 | > 1/100 |
| 1/200 | 1/4,000 | 1/2,000 | 1/500 | > 1/500 | 1/200 |
| 1/400 | 1/4,000 | 1/2,000 | 1/1,000 | > 1/500 | 1/200 |
| 1/800 | 1/8,000 | 1/2,000 | 1/1,000 | 1/500 | 1/400 |
| 1/1,600 | 1/8,000 | 1/1,000 | 1/1,000 | 1/500 | 1/200 |

Antigen was a solution of purified virus 2 mg. per ml.

With dilution of the antigen up to a certain point the end-titre rose; the previous paper recorded a similar effect with sodium chloride M/7 in which the maximum titre was 1/40,000. The results show an inverse relationship between degree of sensitization of antigen and electrolyte concentration required for flocculation.

The antibody optima were very clear in low concentration of sodium chloride, even among close dilutions of antigen showing little differentiation in sodium chloride M/7. In sodium chloride M/56, M/112 and M/224 with serum dilutions from 1/1,500 to 1/8,000 fine tests with numerous preparations of purified virus showed clear optima. Later multiple zones of flocculation appeared. As the virus preparations were presumably pure these zones probably did not indicate the presence of several antigens (as they do with a complex antigenic solution such as serum). Previous work had demonstrated the sensitivity of the system to methods of mixing which might produce artificial multiple zones. In an attempt to overcome this difficulty several procedures were tried: (i) The antigen solution was shaken mechanically for several hours in water or solutions of sodium chloride to aid even distribution before using; (ii) the serum solution was carefully squirted straight into the antigen solution; (iii) or the antigen solution was carefully squirted straight into the serum solution (this is not the usual method for constant-antibody titrations); (iv) the temperature of incubation was varied. None of these modifications eliminated the multiple zones which were most obvious in the more dilute serum and sodium chloride solutions. Numerous preparations of purified virus including one obtained from Cambridge, were included in these tests.

*Serological Tests in Juice from Healthy Tobacco Plants.*

This juice had a conductivity approximately the same as that of sodium chloride M/5; and it should form, obviously, a suitable diluent for the virus. Rapid agglutination occurred in serological tests using this juice. With purified virus (5 mg. per ml.) and serum diluted 1/2,000 the constant-antibody O.R. was 100: 1. In the time taken to read this test no apparent change occurred in a control test of the serum but in 18 hours a small amount of precipitate had appeared. In the juice diluted 1/10 the O.R. was 25: 1 with an end-point at 1/20,480 and no precipitation occurred in the serum control. In sodium chloride M/50, of which the conductivity was approximately the same at that of juice 1/10, the O.R. was 10: 1 and the precipitation end-point 1/5,120. The instability of serum in the presence of juice is possibly responsible for the ready flocculation of the system.

*Effect of Various Salts on the Stability of Virus Suspensions.*

From the results of the following tests salts were chosen for serological work.

Solutions of the selected salts (at pH 7) were mixed with an equal volume of a suspension of purified virus (2 mg. per ml.) and observed in the water-bath at 50° C.

Calcium chloride and magnesium chloride M/10 rapidly precipitated the virus. No precipitation occurred with potassium nitrate or potassium iodide M/20 in 24 hours. The composite buffer solution (0·04M, pH 7) used by Best and Samuel (1936) did not precipitate the virus in two days, but a suspension of 2 mg. of virus per ml. of buffer solution kept at 4° C. for several months precipitated in 24 hours after placing at 50° C. Sodium citrate M/10 slowly precipitated the virus, the precipitation being complete in three days. Sodium acetate, tartrate, benzoate and succinate, and potassium phthalate and malate, all M/10, produced no precipitate in three days.

Best (1940) measured the "critical coagulation concentration" of a number of salts at 30° C. The anion was markedly active. In a list of anions arranged according to the molarity of the critical coagulation concentration, citrate appeared at the top, phthalate and malate in an intermediate position, and the inorganic ions nitrate, iodide and chloride near the bottom.

*Serological Tests with Potassium Phthalate (pH 7).*

After 24 hours at 50° C. a solution of antiserum 1/100 in potassium phthalate M/10 appeared unaltered. The salt was considered suitable for trial in serological tests. Constant-antibody titrations were done in varying concentrations and the results are given in Table 4.

TABLE 4.

*Constant-antibody optima in various concentrations of potassium phthalate pH 7.*

| Serum dilutions. | Optimal ratios at potassium phthalate concentrations of M/14. | M/28. | M/56. | M/112. | M/224. |
|---|---|---|---|---|---|
| 1/1,000 | not done | > 40 : 1 | 30 : 1 | 15 : 1 | 8 : 1 |
| 1/2,000 | 80 : 1 | 60 : 1 | 30 : 1 | 15 : 1 | 8 : 1 |
| 1/4,000 | 30 : 1 | 30 : 1 | 15 : 1 | 15 : 1 | 10 : 1 |
| 1/8,000 | 30 : 1 | 15 : 1 | 10 : 1 | 10 : 1 | about 10 : 1 |

The antigen was a solution of purified virus 5 mg. per ml.

The value of the O.R. fell with falling concentrations of phthalate, and altered with alteration of the concentration of serum. The prozone increased as the phthalate concentration decreased.

For fine tests, close dilutions of several different preparations of purified virus were prepared in potassium phthalate M/56, M/112 or M/224; antiserum was diluted 1/2,000, 1/4,000 and 1/8,000. Only one optimal zone of flocculation occurred in each test but the optimal tube was difficult to pick as the flocculation rapidly extended into the region of antibody excess. Parallel tests with sodium chloride M/28, M/56 and M/112 showed several zones of optimal flocculation. Tests performed by Platt's (1938) method for determining salt optima with bacterial suspensions and their antisera, provided further evidence of the absence of multiple zones in phthalate. This method of mixing had not proved satisfactory in previous tests in sodium chloride M/7. The results of these tests in phthalate were exactly the same as those recorded above. Apparently phthalate reduces the sensitivity of the system to the method of mixing.

Three different preparations containing equivalent quantities by weight (5 mg. per ml.) of purified virus gave the same O.R. in potassium phthalate M/56 with serum 1/4,000; the tube picked as the optimum contained virus solution 1/300 and the O.R. was 13·3 : 1. In potassium phthalate M/10 with serum 1/100 the precipitation end-point of the three preparations was 1/51,200, the same end-point as with sodium chloride M/7.

*Serological Tests with Potassium Malate (pH 7).*

Potassium malate M/10 did not visibly affect antiserum 1/100 during 24 hours at 50° C. Constant-antibody tests were made in various concentrations of this salt; the results appear in Table 5.

TABLE 5.

*Constant-antibody optima in various concentrations of potassium malate, pH 7.*

| Serum dilution. | Optimal ratios at potassium malate concentrations of M/14. | M/28. | M/56. | M/112. | M/224. | M/448. |
|---|---|---|---|---|---|---|
| 1/1,000 | > 40 : 1 | > 40 : 1 | > 40 : 1 | 15 : 1 | 10 : 1 | 3 : 1 |
| 1/2,000 | > 40 : 1 | > 40 : 1 | 20 : 1 | 10 : 1 | 5 : 1 | 2 : 1 |
| 1/4,000 | > 40 : 1 | 20 : 1 | 15 : 1 | 10 : 1 | 5 : 1 | 3 : 1 |
| 1/8,000 | 20 : 1 | 15 : 1 | 10 : 1 | 8 : 1 | 4 : 1 | — |

The antigen was a solution of purified virus 2 mg. per ml.

The value of the O.R. fell with decreasing concentration of the salt or the serum; but the prozone increased as the salt concentration fell.

In fine tests with the three preparations of purified virus used in the last phthalate tests recorded above, only one optimal zone appeared in potassium malate M/56 with serum 1/4,000: the three preparations gave the same O.R., 8 : 1. The optimum was hard to pick as the flocculation rapidly extended into the tubes with excess antibody. In potassium malate M/10 with serum 1/100 these three preparations gave a precipitation end-point of 1/51,200, the same value found in potassium phthalate M/10 and sodium chloride M/7.

In another fine test with one of the purified preparations, potassium malate M/40 and serum 1/6,000 the O.R. was 8·4: 1. After flocculation was complete the tubes were centrifuged and the supernatants tested for antigen by mixing with an equal volume of serum 1/50 and placing at 50° C. overnight; if any appreciable amount of virus was present a precipitate appeared. The supernatants from all antigen dilutions stronger than 1/536 contained some virus; no virus was detected in the supernatants from the neighbouring dilutions 1/536, 1/612, 1/714 and all higher dilutions. The dilution 1/714 which had been picked as the optimum, therefore, lay above the true optimum (i.e. 1/536). This method involving the testing for excess antigen may prove an easier and more accurate one than that of observing the first tube to flocculate.

*Serological Tests with Composite Buffer, Potassium Nitrate or Potassium Iodide.*

With serum 1/1,000 and purified virus (2 mg. per ml.) the constant antibody O.R. fell from more than 36: 1 in composite buffer pH 7, 0·04 M, to 12·5: 1 in composite buffer 0·005 M. Fine tests with a preparation of purified virus 2 mg. per ml. in composite buffer 0·04 M, pH 7, showed several zones of flocculation.

In potassium nitrate and in potassium iodide M/40 fine tests were performed: the details of the tests were identical with those of the fine test described above in potassium malate M/40. Tests for excess antigen were made. In both the iodide and the nitrate tests several zones of flocculation similar to those occurring in sodium chloride appeared. The mixture showing first flocculation in potassium nitrate contained excess antigen and lay above the "equivalent mixture"; in zones appearing later the optimal mixtures contained no excess antigen and lay above the equivalent mixture. In potassium iodide all zones corresponded to mixtures with no excess antigen and lying above the equivalent mixture. All these salt solutions resemble sodium chloride solution in the production of multiple zones which did not appear in potassium phthalate or potassium malate solutions. The results indicate that the anion markedly influences the action of a salt in the serological tests.

## DISCUSSION.

The nature and concentration of the salt used in the test markedly influenced the reaction between the tobacco mosaic virus and its antiserum.

The results observed are probably due, in part at least, to a direct effect on the antigen. The virus may exist in various stages of aggregation depending on the salt and virus concentrations and the pH. (Best, 1937 a and b; Stanley, 1938, 1939).

Hence, in a given salt concentration the alteration of the optimum with alteration of serum concentration may be partly explained in the following way. In a series of antigen dilutions the degree of dissociation of the virus varies from tube to tube. Though by weight the amount of antigen falls off regularly with dilution, the number of antigenic entities depends upon the state of aggregation of the virus. In a certain range, a series of dilutions may alter much less in respect of serological units than in respect of weight of virus; for instance, a two-fold dilution made from a virus solution which flocculated optimally with a certain serum dilution, may provide more than the optimal amount of effective antigen for the same serum solution diluted two-fold. As a result the value of the O.R. will fall. Eventually, with continued dilution, the virus will be completely dissociated; at this stage the number of antigenic entities is likely to be small and the optimum may be influenced by the small number of impacts per unit volume between antigenic particles. This explanation implies that the essential antigenic grouping on the virus molecule is concerned in infectivity and in the linking together of molecules in the paracrystalline fibres.

The increase in the proportion of antiserum in the optimal mixture as salt decreased, may also be due to some extent, to the degree of dispersion of the virus with varying salt concentrations.

Apart from the amount of effective antigen present, the stability of the antigenic entities, which is affected by salt, may also influence the serological reaction. Best (1940) has shown that the stability of virus suspensions decreased as salt concentration increased, as manifested by faster precipitation. In increasing salt

concentration one may reasonably suppose, therefore, that less serum would be required to precipitate the virus and there would be an increase in the amount of serum in the optimal mixture as salt concentration decreased.

The possible action of salt on the serum must not be overlooked. Platt (1938, 1941) indicated that sodium chloride may affect an antiserum. The end-titre of my antiserum for the tobacco mosaic virus increased with increasing salt concentration and the prozone diminished. These effects may, however, be no more than a manifestation of increasing instability of the virus as the salt concentration approached the "critical coagulation concentration."

Platt (1941) suggested that variation in the serological optima with *Br. abortus* indicated S→SR mutation of the organism. The variation in the serological optima with the tobacco mosaic virus may well be an indication of the "roughness" or salt-sensitivity of the antigen.

## SUMMARY.

With the tobacco mosaic virus serological tests were performed with a series of salts and salt concentrations.

The serological optima and the end-titre of the antiserum did not remain constant.

The system appeared less susceptible to variations in technique in potassium phthalate or potassium malate solution than in sodium chloride solution.

It is suggested that the variation in the serological results arises, in part, at least, from the nature of the antigen.

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# STUDIES IN THE METABOLISM OF PLANT CELLS

## 2. EFFECTS OF TEMPERATURE ON ACCUMULATION OF POTASSIUM CHLORIDE AND ON RESPIRATION.

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*(Accepted for publication 1st September, 1944.)*

In an earlier paper (Robertson, 1941) it was suggested that the amount of salt accumulated in the plant cell in a certain time could be explained in terms of the energy necessary for the process; the work done in accumulating salt governed the rate of accumulation. It was suggested that salt accumulation consisted of two stages:

(i) the adsorption of the salt in an outer region of the cell to a constant concentration independent of external concentration, and

(ii) the progressive accumulation of the salt in an inner region of the cell.

On this suggestion it was shown that the total work necessary to accumulate salt from the outer region of constant concentration to the inner region of increasing concentration was proportional to the "salt respiration", i.e., the part of the respiration induced by the application of the salt. It was therefore suggested that the energy necessary for the removal of salt from the outer to the inner region was derived from the salt respiration. Since the concentration in the outer region was considered to be greater than that of the external solution, energy would be required to maintain the concentration in this region but this need be no more than the energy normally necessary to maintain the cell surface. If the salt respiration provides the energy necessary to accumulate the salt, factors which vary salt respiration should vary the amount of salt transferred to the inner region. In the earlier paper such an effect was shown for varying potassium chloride concentrations.

The theory could be tested further by studying the effects of temperature; if the effects of temperature on salt respiration differed from the effects on salt accumulation the theory would be untenable. The purpose of this paper is to record and discuss experiments carried out at four different temperatures.

Apparently no work has yet been published on the effect of temperature on the relationship between salt respiration and accumulation. Hoagland and Broyer (1942) indicate a Van't Hoff coefficient ($Q_{10}$) of about 2 for the accumulation of potassium bromide in roots. The results recorded by Steward (1937) for the accumulation of potassium bromide in potato and artichoke indicate high but variable $Q_{10}$ values; figures are given for total respiration but there is no information as to the magnitude of the salt effect on respiration. The results of Steward and Berry (1943) show that accumulation at different temperatures in potato is clearly different from accumulation in carrot tissue. Neither the linear uptake with time nor the lag period preceding the uptake seen in potato are found in carrot tissue. Further, since Steward and Berry did not investigate the salt respiration (only the total respiration) their observations are not applicable to the discussion in this paper. Some of the earlier results of other workers on the effect of temperature on salt uptake are of doubtful value because of failure to distinguish between ionic exchange and salt accumulation; in some cases they are of no value because of inadequate aeration.

In addition to the effects of temperature on salt accumulation this paper deals with the initial equilibration of the concentration in the tissue with the concentration in the external solution, since this equilibration is relevant to the effects of different temperatures. In the previous paper, only the accumulation which follows this initial equilibration was discussed.

The terminology here is the same as that used in the previous paper.

## MATERIAL AND METHODS.

Carrot tissue cut into discs was prepared in the same way as previously described. Each set of tissue consisted of 80 discs, 1·5 cm. in diameter and 0·1 cm. thick.

The tissue was kept in the apparatus and aerated with $CO_2$-free air in 240 ml. of distilled water until the electrolyte initially lost from the tissue, after washing, had been reabsorbed. The water was then replaced with an equal volume of salt solution and accumulation was measured by determining the progressive changes in the conductivity of the external solution. The $CO_2$ production was measured by passing the gas stream from the tissue through absorbers containing sodium hydroxide solution and measuring the change in conductivity.

One important difference from the technique used previously is to be noted. In the previous experiments no readings were taken in the first ten minutes. In the present experiments the distilled water was poured off the tissue and a rinsing solution of potassium chloride (0·01 M) added; this was poured off immediately and the requisite volume (240 ml.) of 0·01 M potassium chloride added to the rinsed discs. By this technique the initial conductivity of the external solution was that of a 0·01 M solution of potassium chloride and observations were made within the first few minutes.

Two experiments are described; each experiment was done at four different temperatures, 25° C., 19° C., 13° C. and 7° C. Four replicate sets of tissue were used in each experiment; these are called A, B, C and D. In the first experiment the sets were allowed to equilibrate in distilled water at 25° C. and then the salt was added to set A. Some hours later the temperature of all four sets was reduced to 19° C. and after a new steady rate of respiration had been attained the salt was added to set B. The operation was repeated at 13° C. and 7° C. with sets C and D respectively. In this way A experienced four different temperatures after the addition of the salt, set B three, set C two, and set D one only. In the second experiment the technique was similar but the experiment was started at the lowest temperature and therefore one set of tissue experienced the highest temperature at the end, instead of at the beginning of the experiment.

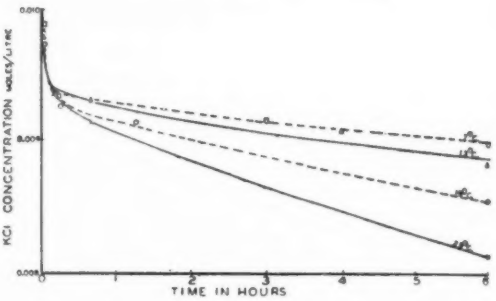


**Fig. 1.** Concentration of KCl in the external solution after the immersion of the tissue at the different temperatures, plotted against time. (Results from experiment II; experiment I gave similar results.)

## RESULTS.

*The Initial Absorption.*

Immediately after the immersion of the tissue in the solution, decrease in the concentration of the external solution took place. The concentration of salt in the external solutions during the first six hours at each temperature is given in Fig. 1. It can be seen that there was an initial rapid equilibration period with a half time of a few minutes which was apparently independent of temperature. This was followed by a period of accumulation during which the different temperatures had different effects.

*Salt Accumulation, Salt Respiration, Ground Respiration.*

After the initial rapid adjustment, accumulation proceeded with a declining rate similar to that noted in the previous paper. The amounts accumulated were obtained by extrapolating the concentration curves shown in Fig. 1 to zero on the time scale and taking this as the concentration from which accumulation began; the change in concentration represents the amounts of

salt accumulated. The justification for this extrapolation will be discussed more fully subsequently. The amounts accumulated at different times and temperatures are shown in Figs. 2 and 3.

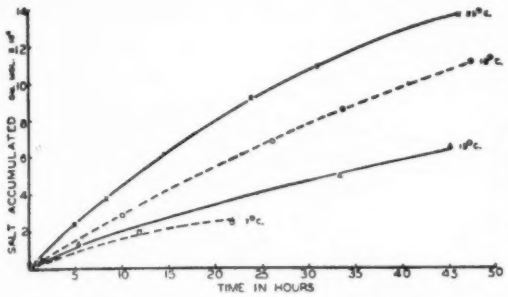


Fig. 2. Amounts of KCl accumulated at different temperatures, plotted against time (experiment I).

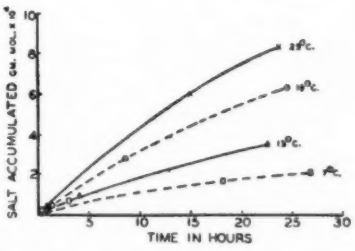


Fig. 3. Amounts of KCl accumulated at different temperatures, plotted against time (experiment II).

TABLE 1.

*Respiration rates ($CO_2$ gm. mol. × $10^4$/hr.).*

| Temperature. | Tissue. | Ground respiration. | Total respiration in M/100 KCl. | Salt respiration. |
|---|---|---|---|---|
| **Experiment I** | | | | |
| 25° C. | A | 0·192 | 0·395 | 0·203 |
| | B | 0·200 | — | — |
| | C | 0·168 | — | — |
| | D | 0·168 | — | — |
| 19° C. | A | — | 0·226 | — |
| | B | 0·104 | 0·240 | 0·136 |
| | C | 0·100 | — | — |
| | D | 0·092 | — | — |
| 13° C. | A | — | 0·094 | — |
| | B | — | 0·102 | — |
| | C | 0·042 | 0·115 | 0·073 |
| | D | 0·051 | — | — |
| 7° C. | A | — | 0·048 | — |
| | B | — | 0·056 | — |
| | C | — | 0·070 | — |
| | D | 0·024 | 0·070 | 0·046 |
| **Experiment II** | | | | |
| 25° C. | A | — | 0·485 | — |
| | B | — | 0·535 | — |
| | C | — | 0·485 | — |
| | D | 0·325 | 0·535 | 0·210 |
| 19° C. | A | — | 0·256 | — |
| | B | — | 0·288 | — |
| | C | 0·170 | 0·312 | 0·142 |
| | D | 0·198 | — | — |
| 13° C. | A | — | 0·165 | — |
| | B | 0·120 | 0·180 | 0·060 |
| | C | 0·103 | — | — |
| | D | 0·103 | — | — |
| 7° C. | A | 0·055 | 0·075 | 0·020 |
| | B | 0·055 | — | — |
| | C | 0·055 | — | — |
| | D | 0·055 | — | — |

The changed temperatures influenced both salt respiration and ground respiration (i.e. the respiration in distilled water). The rates of both salt respiration and ground respiration per set of tissue are given in Table 1; they were steady over the period of the experiment.

The logarithms of the average values for the salt respiration and ground respiration, and the logarithms of the average rates of accumulation during the first 20 hours are plotted against temperature in Fig. 4 (Exp. I) and Fig. 5 (Exp. II). The straight line relation indicates that the rates rise exponentially with temperature.

In each experiment the accumulation of salt in a single set of tissue was examined at the different temperatures. The tissues were set A in experiment I and set D in experiment II. The amounts accumulated by these sets of tissue are shown in Fig. 6. The rate of accumulation was a function of both the temperature and the internal concentration of salt. The results for set A are given over three temperatures only since by the time the last temperature was reached the external concentration of salt was probably low enough to be limiting.

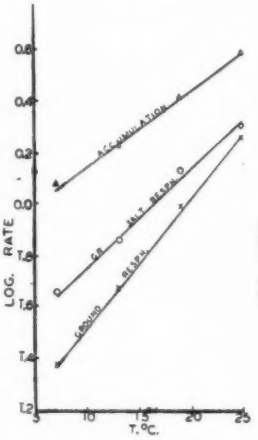


**Fig. 4.** Logarithms of the average values for salt respiration and ground respiration, and of the average rates of accumulation during the first 20 hours, plotted against temperature (experiment I).

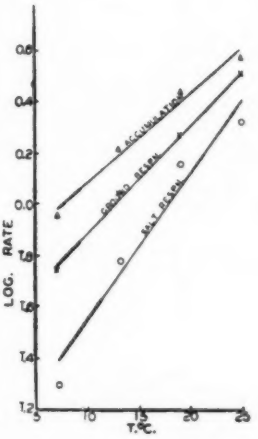


**Fig. 5.** Logarithms of the average values for salt respiration and ground respiration, and of the average rates of accumulation during the first 20 hours, plotted against temperature (experiment II).

## DISCUSSION.

*The Initial Absorption.*

As seen in Fig. 1, there was a rapid decrease in the concentration of the external solution immediately after its addition to the tissue. This decrease in concentration took place within the first 10 minutes. The volume of tissue was 14 ml. and to this was added a solution of 240 ml. It might be expected, therefore, that if the initial potassium chloride content of the tissue were not high there would be a distribution of the salt through the tissue to equality of concentration within and without. The dilution effect to be expected in these experiments would therefore be 5·5 p.c. Since the original concentration of the external solution was 0·01 M, the concentration on dilution with the tissue should be reduced to 0·00945 M. Examination of Fig. 1 shows that this interpretation is consistent with the results. The extrapolations of each of the curves for the concentration of the external solutions meet the ordinate at approximately the same value, between 0·00941 and 0·00944. It is reasonable to assume that some accumulation will take place during these first few minutes when the physical adjustment is taking place, since the surface cells will, during this period, tend to reach equality of concentration quickly and commence to accumulate.

Even though the penetration of the living membrane by potassium chloride is probably not a simple diffusion, energy need not be expended by the cell so long as the movement is from a higher external to a lower internal concentration. Two consequences of this movement along a concentration gradient are:

(a) that the time of reaching equilibrium would be practically independent of temperature ($Q_{10} = 1·2$) if the diffusion were fast, or would be increased markedly by temperature ($Q_{10} = 2–3$) if the diffusion were slow [c.f. Danielli (1943)], and

(b) that the equilibrium concentration reached will be independent of temperature.

There is no distinguishable effect of temperature in the results.

Analysis of the results indicates, therefore, that there is a very rapid adjustment of the concentrations in the cells and that the process of accumulation in which the salt is introduced into the tissue against a concentration gradient begins soon after immersion.

*Salt Accumulation, Salt Respiration, Ground Respiration.*

From Figs. 4 and 5 it can be seen that the effects of temperature on all three processes—salt accumulation, salt respiration and ground respiration—are similar. The increase in rate of all three processes with temperature follows an exponential course with high temperature coefficients. The temperature coefficients are given in Table 2.

TABLE 2.

| | Ground respiration. | Salt respiration. | Salt accumulation. |
|---|---|---|---|
| **Experiment I** | 3·4 | 2·4 | 2·0 |
| **Experiment II** | 2·5 | 3·4 | 2·1 |
| **Average** | 3·0 | 2·9 | 2·1 |

This indicates that all three processes are increased by temperature in the same way as chemical reactions. This is to be expected for both salt and ground respiration and has been suggested from time to time in regard to accumulation. The relation between accumulation and respiration is, however, not simple because at a given temperature the rate of accumulation starts to decrease immediately the process begins but both the ground and salt respiration remain steady in rate over some two days. The decreasing rate of accumulation can be seen in Fig. 6 which shows that the rate of accumulation at any temperature decreases as the amount already accumulated increases.

In the earlier paper it was pointed out that:

(a) energy must be expended to move salt from the outer region to the inner region,

(b) the concentration in the inner region is increasing with time,

(c) the respiration, which is the source of energy, remains constant, and therefore,

(d) assuming a constant fraction of the energy released in respiration to be available, since the energy necessary to move unit amount of salt is increasing, the rate of accumulation must fall.

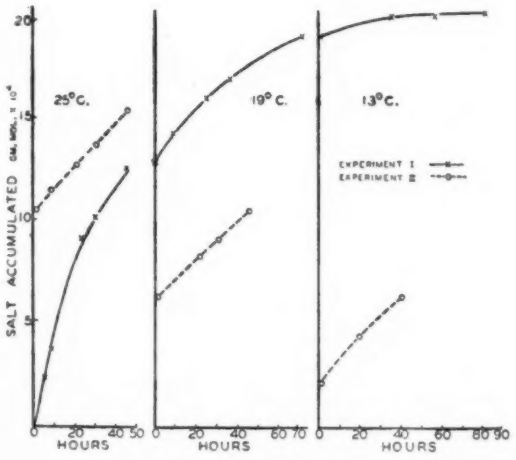


**Fig. 6.** Amounts of KCl accumulated by two sets of tissue at three different temperatures, plotted against time. In experiment I the tissue was initially at the highest temperature and subsequently transferred successively to the lower temperatures; in experiment II the tissue was initially at the lowest temperature and subsequently transferred successively to the higher temperatures.

These experiments can be analysed in the same way. It is assumed that there is built up a region of constant concentration ($C_e$) in the cell and this concentration is considered to be independent of the external concentration (over a certain range). The internal concentration ($C_v$) increases with time as the salt is accumulated.

In the previous paper it was suggested that the concentration in the outer region ($C_e$) could be obtained from comparison of the rates of accumulation at different times and this concentration was used to calculate the work necessary to transfer the salt to the inner region ($C_v$). In the present experiments values for $C_e$ cannot be calculated in this way because the experiments are of shorter duration. For this reason the value of $C_e$ assumed for these experiments is the value used in the earlier paper. It is not considered that reliance should be placed on this value of $C_e$ (i.e. 0·012 M) as an absolute value until further experiments have been carried out. It is convenient, however, to use this figure so that the amount of work can be calculated. A different

figure would alter the estimates of amounts of work but so long as the concentration ($C_e$) can be assumed constant it would not alter the relation between work done and salt accumulated. In any case, the assumption that the potassium chloride in this region has the same activity as that of potassium chloride in water may invalidate the absolute values since activity, and not concentration, must be used in the calculation.

Assuming that the equilibration to $C_e$ in the outer region is rapid and that work must be done to build up the internal concentration ($C_v$), the work necessary to accumulate one gm. mol. of salt at different times at the different temperatures can be calculated from the formula

$$A = RT \log_e \frac{a_v}{a_e}$$

$a_v$ = activity at $C_v$ level.
$a_e$ = activity at $C_e$ level.

The values for $C_v$ are obtained by using the total concentration of salt in the cell and the activity coefficient is assumed to be the same as that of potassium chloride in water. By integrating the curve of amount of work (A) necessary to accumulate unit amount of salt, against the amount of salt accumulated (gm. mol.), it is possible to obtain an estimate of the total work done in accumulation. The results are given in Table 3.

TABLE 3.

| Temperature. | Time (hrs.). | Salt respiration $CO_2$ gm. mol. $\times 10^4$. | $C_v$. | Work/gm. mol. accumulated (cal. $\times 10^4$). | Total work (cal. $\times 10^4$). |
|---|---|---|---|---|---|
| Experiment I | | | | | |
| 25° C. | 0·4 | — | 0·013 | 56 | 6 |
| | 5·0 | — | 0·029 | 492 | 630 |
| | 8·4 | 1·7 | 0·039 | 668 | 1,360 |
| | 24·0 | 5·0 | 0·078 | 1,027 | 6,000 |
| | 31·1 | 6·6 | 0·084 | 1,067 | 6,960 |
| | 46·4 | 9·4 | 0·107 | 1,188 | 10,550 |
| 19° C. | 0·8 | — | 0·017 | 168 | 90 |
| | 10·2 | 1·4 | 0·033 | 555 | 900 |
| | 26·2 | 3·9 | 0·061 | 878 | 3,750 |
| | 33·7 | 5·0 | 0·073 | 964 | 5,240 |
| | 47·6 | 6·8 | 0·091 | 1,088 | 8,000 |
| 13° C. | 1·4 | — | 0·018 | 204 | 70 |
| | 5·4 | 0·4 | 0·023 | 334 | 270 |
| | 33·4 | 2·2 | 0·047 | 725 | 2,000 |
| | 45·2 | 3·1 | 0·058 | 832 | 3,400 |
| 7° C. | 0·2 | — | 0·016 | 161 | 60 |
| | 11·9 | — | 0·025 | 382 | 370 |
| | 21·7 | 1·0 | 0·029 | 460 | 600 |

In the previous paper it was established that when the salt respiration was varied by varying external concentration (the ground respiration being unchanged), the work done in accumulation varied as the salt respiration. In the present paper salt respiration is varied by temperature and it is of interest to determine whether there is any inconsistency in the theory that work done in accumulation and energy liberated in salt respiration are proportional. If the total work done in accumulating is plotted against the salt respiration at each temperature the resulting curve is a straight line (Fig. 7, solid symbols) indicating clearly that the work done in accumulating salt is a constant fraction of the salt respiration, irrespective of temperature. The results of experiment II were similar to those of experiment I. It might be pointed out here that, in the light of the previous work, it is important that there is a linear relation between salt respiration and work done in accumulation; this is of interest though, in this experiment, the relation of work done in accumulating to energy liberated in total respiration would also be linear.

This relationship has been obtained by comparing the rates of accumulation and respiration in replicate sets of tissue at different temperatures. If it is true that work done is proportional

to salt respiration, then the amount of salt accumulated by one set of tissue at different temperatures should be a function of two variables:

(1) temperature and its effect on salt respiration, and

(2) the concentration ratio $\left(\frac{C_v}{C_e}\right)$.

As shown in Fig. 6 one set of tissue in each experiment was exposed to different temperatures while accumulating salt. In each experiment the amount accumulated with time showed the kind of changes which would be expected with changing temperature and increasing internal concentration. The salt respiration has been taken as that obtained by direct measurement, i.e. at 25° C. in the first experiment and at 7° C. in the second experiment; the salt respirations at other temperatures have been derived from this by using the $Q_{10}$ values referred to earlier.

TABLE 4.

| Temp. | Time (hrs.). | Salt respiration rate—$CO_2$ gm. mol. × 10⁴/hr. | Total salt respiration gm. mol. × 10⁴. | $C_v$ | Work/gm. mol. accumulated (cal. × 10⁴). | Total work (cal. × 10⁴). |
|---|---|---|---|---|---|---|
| Experiment I. $Q_{10} = 2{\cdot}4$ | | | | | | |
| 25° C. | 46·4 | 0·203 | 9·41 | 0·107 | 1,188 | 10,550 |
| 19° C. | 120·0 | 0·118 | 18·11 | 0·146 | 1,333 | 17,870 |
| 13° C. | 156·5 | 0·070 | 20·67 | 0·153 | 1,339 | 19,200 |
| Experiment II. $Q_{10} = 3{\cdot}4$ | | | | | | |
| 7° C. | 26·8 | 0·020 | 0·54 | 0·026 | 391 | 520 |
| 13° C. | 67·6 | 0·040 | 2·17 | 0·056 | 810 | 3,230 |
| 19° C. | 114·1 | 0·087 | 6·57 | 0·086 | 946 | 7,300 |
| 25° C. | 160·1 | 0·181 | 14·90 | 0·120 | 1,260 | 13,000 |

If the work done in accumulating is plotted against the total amount of carbon dioxide evolved in salt respiration, the same approximately linear relationship as in the previous paper is observed (Fig. 7, open symbols). Moreover, there is the same departure from the linear relationship at the higher values. This linear relation indicates that the results for an individual set of tissue accumulating at different temperatures can be analysed in terms of work done in accumulating and amount of salt respiration, on the hypothesis that a constant fraction of the energy from salt respiration is expended in raising salt from a region of constant concentration of salt ($C_e$) to a region where the concentration increases with time. Further, since the same value of $C_e$ has been used at all four temperatures, this analysis suggests that $C_e$ is independent of temperature.

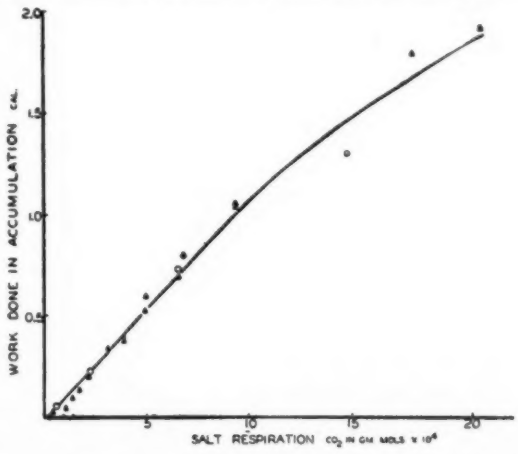


**Fig. 7.** The relation of work done in accumulation to salt respiration. [Solid symbols, results of experiment I (Table 3); open triangles, results for single set of tissue, experiment I, open circles, results for single set of tissue experiment II (Table 4).]

*Relationship to Other Work.*

The results presented in this paper do not conflict with Lundegårdh's theory that salt accumulation is due to liberation of energy in a process of respiration induced by the salt itself. Salt moves against a concentration gradient, and the re-

lation between the liberated energy and accumulation can be explained on the assumption that the energy of the salt respiration raises the salt from a region of constant concentration to a region of increasing concentration. The assumption of a region of constant concentration is consistent with the effects of changing both external concentration and temperature. The salt at a region of constant concentration of this type might correspond with the salt concentration at the surface film of orientated molecules, which is the generally accepted picture of the living membrane (Davson and Danielli, 1943). This layer would be maintained by the ordinary energy consuming processes associated with the formation of the cell surface. Lundegårdh regards the cell surface as consisting of neutral molecules interspersed with others of positive or negative valencies. The negative valencies are probably in great excess of the positive. Under conditions where the cell surface is in contact with distilled water all valencies can probably be pictured as being satisfied by hydrogen or hydroxyl ions. In contact with a solution of a neutral salt, the cations of the salt replace some of the cations at the surface and the anions of the salt replace some of the anions at the surface; replaceable cations are probably in excess of replaceable anions. Provided the external concentration in the solution were not limiting, the concentration of ions bound at the surface would be independent of the small changes in external concentration. If the rate of removal of ions from this layer to the interior of the cell were not great, the pace of the process as a whole would be set by the rate at which energy was available to move the ions through the cytoplasm against the increasing gradient.

It is important to note that the salt respiration does not seem to be limited by the salt concentration in this outer region, since the salt respiration varies although the concentration has been taken as constant at different temperatures. Again, in the earlier paper it was shown that the layer of constant concentration could be assumed to be the same at different external concentrations while the salt respiration differed. These facts suggest that the concentration ($C_e$) of salt at the outer region does not control the salt respiration. Further, there is evidence from both the earlier and the present experiments that the rate of salt respiration remains steady at a given temperature, irrespective of the amount accumulated. It is the presence of salt in contact with the cell which is responsible for the salt respiration. Other evidence (to be published later) indicates that the salt, once accumulated—probably in the vacuole—is incapable of affecting the respiration. Reference has been made to the fact that more exact information must be obtained before the outer region of constant concentration can be defined more precisely. The value of the concentration ($C_e$), as obtained in the previous paper, requires checking for a number of different sets of tissue to determine whether it varies from tissue to tissue. On this value depends the amount of energy necessary to move the salt into the cell and it is important to know whether the energy used in this process is a constant fraction of the energy liberated in the salt respiration process in varied sets of tissue.

The high temperature coefficients and the analysis of the energy relations of the process present no evidence that conflicts with the suggestion that the salt accumulation is dependent on the energy release in salt respiration.

## SUMMARY.

The effects of temperature on the respiration, salt respiration and salt accumulation are investigated in cut carrot tissue. The salt used is potassium chloride and experiments are carried out at four temperatures, 7° C., 13° C., 19° C. and 25° C.

It is concluded that there is a very rapid adjustment after immersion of the tissue in the salt solution resulting in equality of concentration in cells and in external solution. This equilibration is independent of temperature.

The temperature effects on the subsequent salt accumulation can be explained if it is assumed that there is a region of the cell where the salt is maintained in constant concentration and that it is accumulated from this region by an energy consuming process dependent on the energy released in respiration. In view of earlier work it is considered that this energy comes from the salt respiration process. The salt accumulation, salt respiration and ground respiration are shown to have high temperature coefficients. The region of constant concentration is considered to be independent of temperature and not to limit the rate of salt respiration.

*Acknowledgments.* The writer wishes to express his thanks to Professor Eric Ashby, in whose department the work was carried out, and to Professor J. S. Turner, University of Melbourne, for their interest and helpful criticism of the manuscript.

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# PARTIAL COMPOSITION OF TOBACCO MOSAIC VIRUS PROTEIN: THE AMIDE, TYROSINE, TRYPTOPHANE, CYSTINE (PLUS CYSTEINE) AND METHIONINE CONTENTS

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*(Accepted for publication 19th September, 1944.)*

The demonstration that tobacco mosaic virus is a protein (Stanley, 1935, 1936; Best, 1936, 1937; Bawden and Pirie, 1937) has directed attention to its composition in respect to amino acids. A knowledge of the amino-acid composition of viruses in general is of obvious importance as a means of characterizing each virus both as a virus and as a protein, as well as of comparing and classifying the viruses amongst themselves and in relation to other proteins. Combined with a knowledge of the amino-acid make-up of normal plant proteins and the supply of amino-acids normally present in plants, such knowledge may also be expected to throw some light on the method by which the virus multiplies in the plant.

Partial analyses of what we believe to be a particularly pure preparation of the virus protein are recorded in this paper and are compared with results obtained by other workers.

## EXPERIMENTAL.

### *The Virus Protein Preparations.*

For preparation $H2_{40}$, on which most of the work reported here was done, plants of *Nicotiana tabacum*, var. Blue Pryor, were artificially inoculated with a solution of purified tobacco mosaic virus at a concentration of one in three million. The plants were grown in an isolated compartment of a glasshouse during the 1939–40 summer. They were inoculated eleven weeks after germination and harvested 39 days later.

The virus was extracted from the plants as follows. Infected leaves were crushed in a modified fruit juice extractor and the juice so obtained was aerated for 24 hours at room temperature. The virus was then precipitated with ammonium sulphate (to 15 p.c.) and collected by centrifuging. The virus solution obtained by extracting the residue with water was freed from insoluble impurities by centrifuging at about 2,000 g. After five re-precipitations the virus was precipitated by adjusting the pH value of the solution to the iso-electric point of the virus (pH 3·4). The collected precipitate was suspended in water and dissolved by slowly adding N/50 NaOH solution to a vigorously agitated suspension until the pH value was near 7. This solution was centrifuged and treated as described elsewhere (Best, 1940). Altogether, this preparation was re-precipitated iso-electrically nine times. Finally it was extracted three times with absolute alcohol and once with acetone.

Sample IIIm was prepared in a similar way except that instead of aerating the juice it had been held at 65° C. for three hours before the first precipitation with ammonium sulphate. This preparation was re-precipitated four times with ammonium sulphate and four times iso-electrically. The plants from which the virus was extracted were harvested 50 days after being inoculated. The dried preparation contained 16·7 p.c. nitrogen and 0·51 p.c. phosphorus.

### *Methods of Estimation and Results.*

The nitrogen content of preparation $H2_{40}$ was estimated by the Kjeldahl method to be 16·60 p.c., a value for which the random error is believed not to have exceeded 0·05. As determined by the method of Fiske and Subbarow (1925) after ashing with magnesium acetate at 500° C., the phosphorus content was found to be 0·51 p.c. The amide, tyrosine, tryptophane,

cyst(e)ine (cystine plus cysteine calculated as cystine) and methionine contents were estimated by some of the methods described by Lugg (1938c, 1937, 1938a, 1938b). The tyrosine content was estimated by the modified Millon colour reaction for phenols and the tryptophane content by the special colour reaction for indoles, following plain alkali hydrolysis of the protein. The cyst(e)ine and methionine contents were estimated by the "differential oxidation" method [d.o.] and by titration of HI digests [HIt].

Most of the work was done with preparation $H2_{40}$. Very little of preparation IIIm was available, and it was a relatively impure preparation. Its tyrosine content only was estimated.

The amide and amino-acid contents are shown in Table 1, those for preparation $H2_{40}$ being reported on a "nitrogen" as well as a "weight" basis. The random errors of estimation are believed not to have exceeded the following parenthesized quantities: For contents reported on a weight basis; $NH_3$ [amide] (0·02), tyrosine (0·05), tryptophane (0·05), cyst(e)ine [d.o.] (0·01), methionine [d.o.] (0·01), cyst(e)ine [HIt] (0·02), methionine [HIt] (0·02): for contents reported on a nitrogen basis, (0·1), (0·03), (0·04), (0·01), (0·01), (0·02) and (0·03), respectively.

TABLE 1.

*Partial analysis of the virus protein preparations.*

(Values shown in italics are, for reasons explained in the text, considered unreliable or inappropriate to the pure virus protein.)

| | | $NH_3$ [Amide]. | Tyr. | Tryp. | [d.o.]* Cyst. | [d.o.]* Meth. | [HIt] Cyst. | [HIt] Meth. |
|---|---|---|---|---|---|---|---|---|
| IIIm. | As p.c. of protein, by wt. | — | *3·75* | — | — | — | — | — |
| $H2_{40}$ | As p.c. of protein, by wt. | 1·62 | 3·68 | 2·10 | 0·75 | 0·00 | *0·66* | *0·00* |
| | As p.c. of protein N | 8·05 | 1·72 | 1·74 | 0·53 | 0·00 | *0·46* | *0·00* |

* The total-S content of prep. $H2_{40}$ was 0·201 p.c., and the random error of the estimation is believed not to have exceeded 0·003. Sulphate-S could have been present only in quantity too small to be detected in the "differential oxidation" procedure.

## DISCUSSION.

Ross and Stanley (1939a) in earlier investigations of the composition of the tobacco mosaic virus protein, found 3·8 p.c. tyrosine, 2 p.c. tryptophane, 0·6 p.c. cysteine (?) and a trace of methionine. Unfortunately, they supplied little information concerning the methods employed. Their results do not depart markedly from ours. Ross (1942) accounted for 1·9 p.c. of the weight of the protein (containing only 15·9 p.c. N) as ammonia, following prolonged acid hydrolysis. This value would almost certainly include ammonia from other than amide sources. But Ross (1942) quotes a value obtained by Miller (1942) which is only slightly lower (1·8 p.c.) and which might well have been expected to represent the amide content closely on account of the method used. Our value is even lower (1·62 p.c.). The significance of the differences is obscured by the fact that the virus preparations used by these workers were often of unspecified purity and unstated N and P content. Ross (1941) estimated 3·9 p.c. tyrosine in the protein by Lugg's (1937) and Bernhart's (1938) methods applied to alkali hydrolysates. He obtained 2·0 p.c. tryptophane by Lugg's (1937) method applied to alkali hydrolysates and 4·5 p.c. by Shaw and McFarlane's (1938) method, and expressed preference for the latter value. But Shaw and McFarlane's (1938) method is open to the grave objection that a colour reaction for tryptophane is applied to tryptophane and to tryptophane residues in unknown and potentially highly variable peptide combinations. Knight and Stanley (1941) obtained the same value (4·5 p.c. tryptophane) by the same method, and 3·8 p.c. tyrosine by Bernhart's (1938) method. In view of the foregoing, it is perhaps unfortunate that this high value (4·5 p.c.) for tryptophane is usually quoted in reviews and monographs as the correct value, without reservation.

Ross and Stanley (1939b) studied the sulphur-distribution, using Kassell and Brand's (1938) modification of Baernstein's (1936) procedure, and found 0·14 p.c. cystine plus cysteine sulphur, 0·04 p.c. methionine sulphur, and 0·0–0·04 sulphate sulphur. Stanley (1939), however, referring to the work of Ross and Stanley (1939b), stated that of the 0·24 p.c. S usually found, about 0·18 p.c. occurs as cysteine-S and 0·04 p.c. or less as sulphate-S, and that although methionine estimations frequently indicate as much as 0·04 p.c. methionine-S it is doubtful whether the pure virus protein does contain methionine. Ross (1940) estimated the cyst(e)ine and methionine contents of the virus protein by various methods, and concluded that it contains 0·18 p.c. of cyst(e)ine sulphur (estimated by the method of Kassell and Brand (1938) and probably no methionine at all. From Table 1 it will be seen that preparation $H2_{40}$ contained 0·20 p.c. sulphur, all of which (according to the results obtained by the "differential oxidation" method) must have been present as cyst(e)ine sulphur.

The cyst(e)ine content of preparation $H2_{40}$ estimated by titration of HI digests (indicating nearly 0·18 p.c. of cyst(e)ine sulphur) was rather lower than that estimated by the "differential oxidation" method. The significance of differences between values obtained by these two methods has been discussed elsewhere (Lugg, 1938b; 1939). Although virtually no humin was produced during the HI digestion, the virus protein is a nucleo-protein containing carbohydrate in the nucleic acid portion of the molecule and the carbohydrate could have caused under-estimation of the cyst(e)ine. Even with recrystallized edestin, Lugg (1938b) encountered a discrepancy of about 4 p.c. in the same direction between values estimated by the two methods.

It is believed that the value obtained by the differential oxidation method is the more reliable although the method itself is the less specific one. Stanley and Lauffer (1939), using Greenstein's (1938) porphyrindin dye titration procedure, estimated that the virus protein contained 0·76 p.c. cysteine. This is in close agreement with the value obtained by the "differential oxidation" method and reported in Table 1.

## SUMMARY.

Partial analyses have been made of the tobacco mosaic virus protein. Of the protein nitrogen (the purified protein contained 16·60 p.c. N), 8·05 p.c. was estimated to occur as amide, 1·72 p.c. as tyrosine, 1·74 p.c. as tryptophane, 0·53 p.c. as cyst(e)ine and 0·00 p.c. as methionine. Expressed as a percentage by weight of the protein, the corresponding values are: 1·62 p.c. ammonia (amide), 3·68 p.c. tyrosine, 2·10 p.c. tryptophane, 0·75 p.c. cyst(e)ine, 0·00 p.c. methionine. No sulphate-S could be detected.

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# STUDIES ON A FLUORESCENT SUBSTANCE PRESENT IN PLANTS[1]

## 2. ISOLATION OF THE SUBSTANCE IN A PURE STATE AND ITS IDENTIFICATION AS 6-METHOXY-7-HYDROXY 1:2 BENZO-PYRONE.

by RUPERT J. BEST

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*(Accepted for publication 19th July, 1944.)*

The discovery of a fluorescent substance in certain virus-infected and healthy plants was reported in a previous paper (Best, 1936a) and some properties of the substance, determined on samples of the crude extract, were described. The present paper describes the isolation of the substance in the form of pure crystals and its identification as a substituted benzo-pyrone.

### ISOLATION OF THE SUBSTANCE.

*Source of Material.*

A survey of the occurrence of the fluorescent substance in T.S.W.-susceptible plants available locally showed that tobacco plants contained as high a concentration of the substance as any tested by the author. An investigation into its distribution within the plant showed that it accumulated in the roots. One sample was worked up from leaf material only and another sample from roots only. The two samples were found to have the same melting point and properties. Root extracts were simpler to handle and consequently the roots were used as primary source.

Batches of 100 plants of *Nicotiana tabacum* (var. Blue Pryor) were topped at the stage when they would normally be used for quantitative primary lesion work (about 5 to 8 weeks after germination). They were then inoculated with a suspension of tomato spotted wilt virus. The resulting lesions were allowed to develop to a late stage. All secondary growth was removed as it appeared. This and topping were done because these treatments favoured accumulation of the substance in the roots. From observations, it was concluded that the substance formed in the leaves moved to the roots by way of the vascular system and concentrated there. Examination of the roots under filtered ultra-violet light revealed a lower concentration of the substance in the roots of parallel lots of plants which had not been topped or from which the secondary growth had not been removed.

The time between inoculation and harvesting varied with the rate of development of lesions which, in turn, was mainly dependent on temperature. The plants were usually ready to harvest in from 1½ to 2½ weeks after inoculation. They were then removed and the roots were quickly washed in cold water.

*Method of Extracting the Substance.*

Tests had shown that the partition of the substance between water and chloroform varied with the pH value of the aqueous solution. In acid solutions the substance passed from the aqueous solution to the chloroform, and could be extracted from chloroform by aqueous media with pH values above 8. At high pH values the substance undergoes an irreversible change. A pH value of about 10 was found to be most suitable for transferring the substance from the chloroform extract back to water without causing irreversible change. The following is the procedure which was finally adopted.

The washed roots are transferred to a suitable bottle and enough of a dilute solution of sulphuric acid (about 0·05 N gave good results) is added to wet them thoroughly and to submerge from two-thirds to three-quarters of their bulk. After about an hour's contact with the acid, enough chloroform is added to raise the level of the sulphuric acid so that it completely covers the roots. The whole is allowed to stand till next day, when the liquids are poured off and separated. The aqueous layer is re-extracted with chloroform another two or three times and the chloroform extracts are bulked.

The fluorescent substance is next removed from the chloroform by extraction with an aqueous solution of pH about 10, a solution of ammonium hydroxide (M/100) serving well for the pur-

[1] Glasshouse facilities and part of the cost of this investigation were provided by the Council for Scientific and Industrial Research (Australia).

pose. Two or three extractions, bringing the total volume of extract up to that of the chloroform being extracted, usually suffice.

The water extract is then acidified by adding just enough normal sulphuric acid to bring the pH value to 3 or 4 (the exact pH value does not matter). The end-point can be very easily distinguished in daylight by the sudden disappearance of the intense blue fluorescence. The substance is again extracted from the acidified aqueous solution by shaking with chloroform. The chloroform extracts are bulked and dried with neutral calcium chloride lumps (the neutral form must be used as the ordinary form gives an alkaline solution and considerable loss of the substance is caused by the passage from the chloroform to the moist alkaline surface of the calcium chloride lumps). The dried chloroform extract, after being filtered through a suitable filter paper (541 Whatman) is concentrated by distilling off the chloroform over a water bath until only about 100 ml. remain. The concentrate is transferred (with chloroform washings) to an evaporating basin and is evaporated to dryness on a slow water bath, care being taken not to overheat towards the end. The cream-coloured residue is then dissolved in water at or near the boiling point and is recrystallized from water. If this last operation is done in a centrifuge tube the crystals may be easily collected by centrifuging, draining and finally drying in a desiccator. The crystals must be stored in the dark.

All operations from the time the roots are washed to the final crystallization must be carried out in the dark or in a subdued light. The substance is sensitive to light and the colour of the final product depends on the amount of light admitted during the process. Too long heating also causes the colour to darken.

## PROPERTIES AND CHEMICAL COMPOSITION.

*Physical Properties.*

The substance crystallizes from water as pale canary-yellow needles. Crystals from chloroform are paler in colour. The photo-micrograph reproduced in Fig. 1, besides showing the form of the crystals, illustrates their fluorescence when irradiated with ultra-violet light. The *melting point*, as determined with a short-stemmed thermometer, was 206° C.

**Fig. 1.** Photomicrograph of crystals of the fluorescent substance (from chloroform) photographed by the light of their own fluorescence. The source of illumination was filtered ultra-violet light (3,200–4,100 A) focussed on to the crystals (oblique illumination). An aesculine filter was placed on top of the microscope ocular to filter out any reflected ultra-violet light. (× 103).

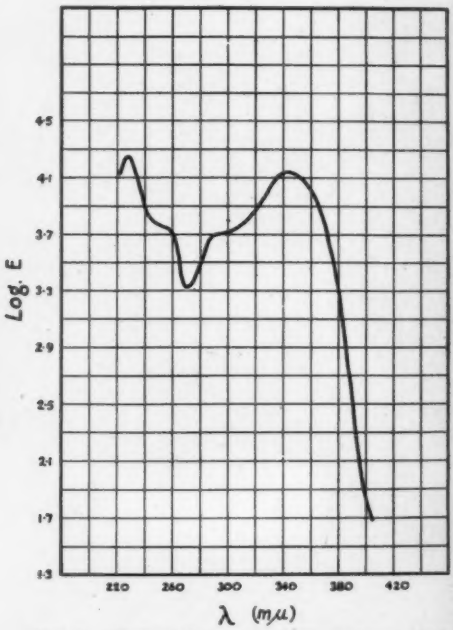



**Fig. 2.** Ultra-violet absorption spectrum of the fluorescent substance (6–Methoxy–7–hydroxy 1: 2 benzo–pyrone) in ethyl alcohol.

The *solubility* of the substance in water at 17° C. was 0·20 mg. per ml. of saturated solution (1 in 5,000 or M/1,000).

Samples of the substance obtained from healthy tobacco plants had the same M.Pt., chemical composition and other properties as samples prepared from infected plants, but the substance occurred in greater amounts in the latter.

The most characteristic property of the substance is its extremely strong fluorescence when its solutions in polar solvents are irradiated with ultra-violet light. This property was made use of in tracing the partition of the substance between various solvents under various conditions and led ultimately to its isolation. Under ultra-violet light aqueous solutions show a blue fluorescence which is stronger and lighter at higher pH values. In daylight aqueous solutions of the pure substance at pH values near 7 fluoresce moderately and under the same conditions alkaline aqueous solutions show a strong blue fluorescence, whereas in acid solutions the fluorescence is scarcely perceptible.

The ultra-violet absorption spectrum is plotted in Fig. 2.[2]

*Chemical Composition and Properties.*

Qualitative tests revealed the absence of common elements other than C, H and O. An (OH) group was indicated by the green colour produced by a solution of $FeCl_3$. A solution of the substance in chloroform decolorized a solution of bromine in $CCl_4$ without evolution of gas. The substance reduced Fehling's solution and ammoniacal silver nitrate. The addition of oxidising agents such as chloramine T, bromine water and $KMnO_4$, under certain conditions, produced an intense blue colour. The colour changed to yellow on rendering the solution alkaline, and reverted to blue on re-acidification provided it was not allowed to stand too long at a high pH value. When halogen is added to a weakly alkaline solution of the substance a yellow colour is produced which, on acidification, turns blue.

As the yield of pure substance was about 0·1 mg. per plant, analyses had to be done on a micro scale. Micro analysis of four samples prepared from four different harvests gave the following results.[3]

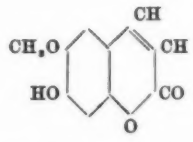
TABLE 1.

| | Range (p.c.). | Mean (p.c.). |
|---|---|---|
| C | 62·3 –62·6 | 62·5 |
| H | 4·15– 4·27 | 4·18 |
| O (by diff.) | | 33·3 |
| Mol. wt. | 192–201 | 195 |

The above analyses clearly indicate an empirical formula of $C_{10}H_8O_4$. This, together with the properties and reactions of the substance suggested a coumarin derivative. Methoxy determinations gave values ranging from 16·10 to 16·18 p.c. with a mean value of 16·15 (compared with 16·15 theoretically required for a methoxy-hydroxy benzo-pyrone).

## DISCUSSION.

A comparison of the observed figures with theoretical requirements as tabulated below leaves no doubt that the substance is 7–hydroxy–6–methoxy 1 : 2 benzo-pyrone (Scopoletin).




| | C(p.c.) | H(p.c.) | O(diff.) | ($OCH_3$) | Mol. Wt. | M.Pt.(°C.) |
|---|---|---|---|---|---|---|
| Found | 62·5 | 4·18 | 33·3 | 16·15 | 195 | 206 |
| Scopoletin requires | 62·5 | 4·20 | 33·3 | 16·15 | 192 | 204* |

*(Figures in the literature vary from just under 200° to 204°).

[2] I am indebted to Mr. R. G. Cooke of the Johnson Chemical Laboratories, University of Adelaide, for determining this curve and to Professor A. K. Macbeth for providing the necessary facilities.

[3] I am indebted to Dr. G. Burger of the University of Adelaide for all micro-analyses, including methoxy determinations.

The substance has been described in the literature as a constituent of certain pharmaceutical extracts, and as a normal constituent of the bark of *Scopolia japonica*, the roots of *Gelsemium sempervirens* and a number of other plants (Wormley, 1870; Eijkman, 1884; Schmidt, 1898). It has not previously been recorded in tobacco. Its synthesis was first effected by Head and Robertson (1931). It is variously known as scopoletin, chrysotropic acid, gelsemic acid, $\beta$ methyl aesculetin, and coumarinic lactone.

Scopoletin and its glucoside scopolin have been recorded as occurring together in some plants. It was important to make sure that the final product was not just a hydrolysis product of the glucoside obtained during the manipulations. For this reason the first samples prepared were extracted from plant material by water only. The separation of the substance from the aqueous solution so obtained was effected by the method described earlier in this paper.

The form of the ultra-violet absorption spectrum (Fig. 2) is of interest. There are two maxima, one at $\lambda$2285Å, log E 4·25, and another at $\lambda$3435Å, log E 4·14. No record could be found in the literature of the absorption curve of this substance although Tasaki (1927) records those of coumarin and a number of its derivatives. According to Tasaki, coumarin showed two absorption maxima at 3200Å and 3600Å, but substances formed by introducing one or two hydroxy groups into the benzene nucleus showed only one maximum.

Fluorescent substances absorbing in the ultra-violet often exert photo-dynamic action. The circumstance that abnormal accumulation of this substance in the plant is the result of injury (Best, 1936) led Dr. L. B. Bull of the Division of Animal Health and Nutrition, Council for Scientific and Industrial Research, to test the substance for such action. White mice, whether injected intra-peritoneally or subcutaneously with up to 2·5 ml. of a saturated aqueous solution of the substance prepared by the author, showed no photo-sensitization on exposure to unfiltered sunlight.

*Possible Mode of Action of the Substance on the Virus in the Plant.*

Noack (1920) considered that all organic fluorescent substances have the same type of physiological action and states that he has obtained a photo-chemical oxidation of certain plant chromogens to dyestuffs by means of aesculin, a 6-glucoside of 6 : 7 di-hydroxy 1 : 2 benzo-pyrone. The action was essentially one of oxidation and was prevented by sodium sulphite and other reducing salts.

Sieburg (1921) showed conclusively that scopoletin belongs to the exceptional group of fluorescent substances which act as optical desensitizers on erythrocytes. It further protects the erythrocytes against the accelerated haemolytic action of saponins usually brought about by irradiation, and it also has a protective action on the straight-out chemical photo-dynamic reaction KI $\times$ light $\rightarrow$ I.

In a typical lesion caused by the virus of tomato spotted wilt on tobacco, the concentration of fluorescent substance is highest where the concentration of active virus is greatest. If, as seems likely, virus activity breaks some link in a chain of reactions in which this benzo-pyrone is an intermediate, and so causes it to accumulate, then no other explanation of the above observation is required.

However, the circumstance that virus and scopoletin occur together raises the question as to whether scopoletin has any direct effect on the virus in the plant. If Noack's conclusion is correct the substance may well act as an inactivant in the presence of light. It has been shown (Best, 1935, 1936b) that light depresses the development of tomato spotted wilt virus in inoculated tobacco leaves. This is what might be expected if photo-chemical oxidation of scopoletin takes place in the presence of light.

In view of Sieburg's results we must also consider the possibility of the fluorescent benzo-pyrone actually protecting the virus against inactivation, but this very action would be expected to result in the production of oxidation products which would act as inactivants for the virus.

Little is known about the function of this benzo-pyrone in the normal metabolism of the plant. Its presence in healthy plants may be as an excretion product. But the observations referred to earlier in this paper on the effect of topping and secondary growth on the accumulation of the substance suggests that it may be concerned with growth. Its concentration in the endodermal cells is also suggestive and will be discussed in relation to function in a later paper in this series describing the distribution of the substance within the tobacco plant.

## SUMMARY.

The fluorescent substance previously described as being produced in unusually large quantities in certain plants as the result of virus activity has been isolated in the form of pure crystals.

The substance can be extracted from acidified aqueous solutions by chloroform and from its solution in chloroform by weakly alkaline aqueous solutions (around pH 10).

A method is described of obtaining the substance from young decapitated tobacco plants after inoculating them with the virus of tomato spotted wilt and allowing the substance to accumulate in the roots.

The average amount recovered in pure crystalline form from such plants was of the order of one-tenth of a milligram per plant. Older, larger plants gave a greater yield.

Healthy plants gave smaller yields of the same substance.

The substance was shown to be 6–methoxy–7–hydroxy 1:2 benzo-pyrone (Scopoletin).

The ultra-violet absorption spectrum shows two maxima, one at λ2285Å, log E 4·25 and another at λ3435Å, log E 4·14.

The possible mode of action of the substance on the virus in the plant is discussed.

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# THE ROLE OF BORON IN PLANT METABOLISM

## 1. BORON IN RELATION TO THE ABSORPTION AND SOLUBILITY OF CALCIUM.

by MALCOLM E. SMITH[1]

(From the Division of Plant Nutrition, University of California).

*(Accepted for publication 3rd October, 1944.)*

This paper is the first of a series dealing with certain of the experiments conducted in the course of an investigation whose aim was to arrive at a better understanding of the metabolism of boron in plant life. In contrast with previous studies of boron in plants, an attempt was made to approach the problem from as many directions as possible. To do this, a large number of completely different types of experiment was designed. Then each experimental approach was explored only until it was felt that the final solution did not lie in that direction, when it was abandoned. For this reason many of the experiments will appear incomplete.

The earlier studies were devoted to the absorption and distribution of boron and calcium. There are often striking similarities between the symptoms of deficiency of these two elements, which have led to frequent suggestions of an interdependence of their metabolism (Brenchley and Warington, 1927; Wolf, 1939; Swanback, 1939, etc.). Skolnik (1934) observed a decrease in the absorption of calcium, phosphate and nitrogen, and an increase in potassium uptake, in boron-containing solutions. Conflicting evidence was presented by Rehm (1937), who found that boron promoted the absorption of cations, and inhibited that of anions. According to Minarik and Shive (1939), the maximum amount of calcium in the leaves of soya beans occurred in those plants which received optimum concentrations of boron. Recently Parks, Lyon and Hood (1944) have greatly clarified the confused situation by determining the influence of boron, over the course of several months, upon the concentrations of thirteen different elements in tomato leaflets. Their conclusion with respect to calcium absorption coincided with that of Minarik and Shive mentioned above. Previously an attempt had been made by the writer (Smith, 1943) to determine whether an effect upon the absorption of calcium could be detected within a few hours after addition of boron. Some short-time experiments, in which the calcium or radioactive strontium was determined in the exudates from cut stems of tomato plants at hourly intervals, indicated that boron had no apparent effect upon calcium absorption.

Attention was then turned to the distribution of boron and calcium within the cell. Marsh and Shive (1941) had found that the soluble calcium in the tissues of the corn plant was determined not by the total calcium but by the boron content of the substrate. It therefore seemed desirable to investigate the nature of the so-called soluble and insoluble boron and calcium in the cell.

### EXPERIMENTAL.

Estimation of boron and calcium, in the various parts of the living cell, would provide information contributing towards a knowledge of their function. There is no satisfactory method for this estimation. The process of dissecting individual cells is far too laborious. A "radiograph" of a single cell of a higher plant cannot be obtained because of the comparatively large grain size of photographic film. Nevertheless it seemed worthwhile to attempt even a very

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approximate determination of the distribution of these elements, involving the use of killed tissue, and the treating together of many cells and cell types instead of individual cells, yielding preparations whose original location was inevitably a matter of surmise.

*Analytical Methods for Boron and Calcium.*

Boron determinations were conducted in duplicate or triplicate, following a modification of the procedure of Berger and Truog (1939). Difficulty was encountered with certain plant materials, owing to loss of boron during evaporation and ashing. Exudate samples tended to lose boron if ashed for several hours at 450 to 500° C., yet long ashing at this temperature was necessary to remove the last traces of nitrate, which interfered with the determination. Generally it was satisfactory to carry out the evaporation in the presence of calcium hydroxide, followed by ashing for 4 hours at 450° C.

In the case of leaf fractions, a little more calcium hydroxide was added than necessary to make the mixture alkaline to phenolphthalein. It was then possible to dry at 100° C., and ash for 4 hours at 430° C. (± 10°), without any loss of boron. Whenever calcium hydroxide was employed, correspondingly greater amounts of sulphuric acid were used in trituration of the ash.

Calcium was determined in duplicate samples according to the method of Kirk and Tompkins (1941).

*Influence of Boron upon Calcium Distribution in Leaf Cells of Tomato Plants, as indicated by Radioactive Strontium.*

Radioactive strontium[2] was employed to indicate the location of the calcium in the cell, on the supposition that strontium translocation might parallel that of calcium, as it apparently does in animals, even though the chemical properties of these two elements are appreciably different. In one case, actual calcium determinations were made also, to provide a comparison with the strontium concentrations.

TABLE 1.

*Influence of boron on the distribution of radioactive strontium in leaf cells of tomato.*

| | | Boron deficient. | Normal leaves. | |
|---|---|---|---|---|
| | Fraction. | Percentages of total Sr. | Percentages of total Sr. | Percentages. of total Ca. |
| A | "sap" | 10·7 | 5·7 | 12·0 |
| B | 1st "sap-wash" | 9·4 | 3·4 | 5·2 |
| C | 2nd "sap-wash" | 5·5 | 5·7 | 7·1 |
| D | filtrate from I | 3·9 | 6·8 | 7·9 |
| E | "aqueous extract" | 5·1 | 1·7 | 3·7 |
| F | 1st "chloroplasts" | 4·5 | 3·4 | 10·2 |
| G | 2nd "chloroplasts" | 0·8 | | |
| H | 3rd "chloroplasts" | 0·2 | | |
| I | "protein" | 0·2 | 0 | |
| J | "acid extract" | 57·6 | 71 | 52·5 |
| K | 2nd "acid extract" | 1·6 | | |
| L | residue | 0·3 | 2·4 | 1·4 |

Note. For normal tomato roots, the strontium distribution was as follows: "sap", 5 p.c.; sum of "sap-washes", 22 p.c.; filtrate from grinding and squeezing through muslin (contained protein, etc.), 10 p.c.; fraction obtained by extraction of the residue of wall material with very dilute acid, 62 p.c.

Marglobe tomato plants were reared in culture solution, containing 0·1 p.p.m. of boron, until they attained a height of two feet. At this stage they received a boron deficient solution. to which was added radioactive strontium lactate. Two weeks later the leaves were harvested and frozen. Fractionation of the thawed leaf tissue was carried out as follows:

A. Sap obtained by gentle pressing; B. First sap-wash (prepared by allowing the residue from A to imbibe water, followed by squeezing); C. Second sap-wash; D. Clear orange-coloured filtrate, obtained by grinding the residue from C in a Waring Blendor with 500 ml. of water, squeezing the mixture in washed muslin, and then removing F, G, H and I from the resulting liquid; E. Filtrate after regrinding with water the residue from D, and squeezing through muslin (E contained some chloroplast material, protein, etc., but not nearly as much as the first filtrate through muslin); F. Precipitate of chloroplasts and starch, resulting from low-speed

[2] Obtained through the courtesy of Dr. M. D. Kamen, Radiation Laboratory, University of California.

centrifugation of the first filtrate through muslin; G. Main chloroplast fraction, thrown down by centrifuging at a higher speed than for F; H. Chloroplast material, separated by recentrifuging for twenty minutes at the higher speed; I. Greenish-grey precipitate of protein, etc., produced in the supernatant liquid from H by the addition of trichloracetic acid; J. Acid-soluble fraction, prepared by regrinding in the Blendor the residue from E with very dilute hydrochloric acid, and then squeezing through muslin; K. Solution obtained by extraction of the residue from J with 3 N. hydrochloric acid for several hours on the steam-bath; L. Residue from K—mostly wall material.

Radioactive strontium was determined in the above fractions, and the amounts expressed as percentages of the total count of strontium for the whole leaf sample (Table 1). Several fractions appeared to be of little interest, and were therefore included with other fractions in subsequent investigations.

For comparable tomato plants supplied with 0·5 p.p.m. of boron, the above procedure was repeated. Fractions F, G and H were grouped together under the heading of F, while K was eliminated so that L represented the sum of K + L in the previous system.

*Boron and Calcium Distribution in Leaf Cells of Squash Plants.*

Leaves of Hubbard squash were fractionated, and analyses conducted for both calcium and boron. On theoretical grounds, it was expected that boron in wall material would be much more soluble in weak alkali, than in the weak acid used for calcium extraction. A preliminary experiment, although unreliable due to loss of boron during evaporation and ashing, indicated that practically none of the boron in the wall material was extracted by grinding with very dilute potassium hydroxide, whereas the procedure used previously for removing calcium was most effective. This would suggest that the so-called insoluble boron was present in some carbohydrate complex, which was readily hydrolyzed by weak hydrochloric acid solutions, but not by dilute alkali.

TABLE 2.

*Distribution of boron and calcium in cells of squash leaves from plants supplied with 0, 0·5, and 10 p.p.m. of boron in the culture solution. (The starting material for each fractionation was 150 gm. of fresh leaves.)*

| Fraction. | | | A | B | C | D | F | G | H | I | J | L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Approximate designation. | | "Sap". | | "Sap-wash". | Filtrate from I. | | "Chloroplasts". | | "Protein". | Acid extract of "wall". | Residue. |
| I | γ B/gm. dry wt. of each fraction | — B | 0·8 | | 3·9 | 8·3 | | 0·13 | | 1·8 | 10·5 | |
| | | normal | 24 | | 10 | 30 | | 1·2 | | 2·5 | 40 | |
| | | high B | 200 | | 290 | 510 | | 50 | | 80 | 200 | |
| | mg. Ca. per gm. dry wt. of each fraction | — B | 10 | | 3·7 | 21 | | 4·4 | | 1·5 | 44 | |
| | | normal | 16 | | 6 | 18 | | 6 | | 1·2 | 39 | |
| | | high B | 24 | | 29 | 40 | | 10 | | 2·3 | 47 | |
| II | B in each fraction as a percentage of total B in 150 gm. of leaves | — B | 3·2 | | 12·8 | 12·0 | | 0·4 | | 2·7 | 55·1 | 13·8 |
| | | normal | 19·2 | | 6·8 | 17·0 | | 0·5 | | 2·1 | 50·0 | 4·4 |
| | | high B | 19·4 | | 13·6 | 27·3 | | 2·8 | | 1·8 | 32·7 | 2·4 |
| | Ca in each fraction as a percentage of total Ca in 150 gm. fresh leaves | — B | 10·7 | | 3·1 | 7·7 | | 3·7 | | 0·6 | 71·6 | 2·6 |
| | | normal | 11·1 | | 3·9 | 8·7 | | 2·4 | | 0·8 | 72·3 | 0·8 |
| | | high B | 10·9 | | 6·4 | 10·0 | | 2·6 | | 0·25 | 67·7 | 2·0 |
| III | γ B/gm. dry wt. of total sample of leaves | — B | 0·16 | | 0·64 | 0·59 | | 0·02 | | 0·14 | 2·7 | 0·68 |
| | | normal | 3·5 | | 1·3 | 3·1 | | 0·10 | | 0·38 | 9·1 | 0·80 |
| | | high B | 34 | | 23 | 47 | | 4·8 | | 3·2 | 56 | 4·1 |
| | mg. Ca/gm. dry wt. of total sample of leaves | — B | 2·1 | | 0·60 | 1·5 | | 0·72 | | 0·12 | 14 | 0·5 |
| | | normal | 2·4 | | 0·82 | 1·8 | | 0·49 | | 0·18 | 15 | 0·13 |
| | | high B | 4·0 | | 2·3 | 3·6 | | 0·94 | | 0·093 | 24 | 0·73 |

Boron-deficient, normal, and high-boron Hubbard squash plants were grown in culture solution under comparable conditions. Normal plants received 0·5 p.p.m. of boron in the culture solution, high-boron plants 10 p.p.m. Four weeks from planting, when definite boron deficiency symptoms were evident, young to fully grown leaves were harvested from twenty-four plants in each treatment. Old leaves were discarded. Freezing and fractionation were carried out in almost the same fashion as for the tomato leaves, using samples of fresh weight 150 gm.

In boron-deficient squash leaves the material was brought to room temperature, pressed gently, and the issuing liquid labelled sap, A. 70 ml. of water were added to the residue, which was washed by alternate pressing and reabsorption. This was repeated with three more portions of water, and the four washes considered together as sap-wash, BC. To the residue were added 400 ml. of water, and the mixture thoroughly ground in a Waring Blendor. The liquid obtained, after squeezing through washed muslin, was centrifuged for twenty minutes, to give a chloroplast fraction FGH. The supernatant liquid, on treatment with 12 ml. of 10 p.c. trichloracetic acid, yielded a precipitate of protein, etc., I, and a clear yellow liquid D. The residue of wall material, from the first squeezing through muslin, was blended with 300 ml. of water and 15 ml. of concentrated hydrochloric acid. Squeezing this mixture through muslin gave an acid extract J, and a residue of wall material L.

Profiting from the experience of the previous experiment, aliquots for boron determination were made alkaline to phenolphthalein, by the addition of calcium hydroxide, before evaporation and ashing.

With normal squash leaves some modification in the above procedure were unfortunately necessitated by the different nature of the chloroplasts. However, as the analyses show, the changes caused no important inaccuracy in the results. Upon centrifugation to produce fraction FGH (''chloroplasts''), considerable chloroplast material remained in the supernatant liquid. It could not be removed by long centrifugation, or by filtration through a number 42 Whatman paper. On microscopic examination, these particles appeared to be small fragments of chloroplasts. They were precipitated by the trichloracetic acid treatment, and are therefore included in fraction I (''protein'').

In high-boron squash leaves fractionation was carried out precisely as for normal leaves. Results of the boron and calcium analyses are given in Table 2.

## DISCUSSION.

In the case of the tomato leaves in Table 1, boron deficiency resulted in an increase of soluble strontium in the cell, at the expense of the insoluble strontium of the cell wall. However, calcium analyses indicated that the strontium did not quantitatively represent calcium distribution. It would appear that strontium was immobilized in the cell walls to some extent (especially in normal leaves) resulting in lower than the expected concentrations in vacuole and chloroplasts. This was suggestive of a detoxification mechanism, the strontium being rendered innocuous by combination with the solid substance of the cell wall.

On the basis of fresh weight, the high-boron squash leaves of Table 2 contained considerably more calcium than normal, while in terms of dry weight, the boron-deficient material was lower in calcium than normal. It is therefore difficult to find a reliable basis for comparison of the calcium content of fractions from the three types of leaf material. Concentrations in terms of dry-weight are of doubtful significance, on account of the undetermined fates of the hydrochloric acid and trichloracetic acid, which were responsible for part of the dry-weight of fractions I (''protein''), D (filtrate from I), J and L (''wall''). Nevertheless the values as they appear in part (i) of Table 2 are probably fairly accurate, with the possible exception of those for fractions D and I.

If the amounts of boron and of calcium, in each fraction, are expressed as percentages of the total amount of the element in the original leaf sample (as in part ii of Table 2) they do not show evidence of the close relationship between soluble boron and soluble calcium found by Marsh and Shive (1941) for the corn plant. However, when the analyses are expressed in the same terms as employed by these investigators (part iii of Table 2) the results are in accord with theirs, if it be considered that fraction A corresponds to their ''soluble boron'' and ''soluble calcium'', as indeed it should. It is nevertheless well to emphasize that both here, and in the above-mentioned study of the corn plant, the insoluble calcium (in terms of total dry weight) also increased with increasing boron concentrations.

By a comparative examination of parts (i), (ii) and (iii) of Table 2, it is possible to reach some general conclusions:

*Fraction A—"vacuolar sap".* No great differences occurred in the calcium of the vacuole, but in boron deficient leaves the vacuolar boron was reduced to a very low level.

*Fraction BC—"sap-wash".* Probably this extract contained solutes washed out of the cytoplasm, as well as a small amount of vacuolar sap. Also it might have contained the soluble pectic substances, besides some sugars and organic acids which were not washed out of the cytoplasm by the vacuolar sap on its removal. Boron and calcium associated with proteins would be expected to be leached out of the cytoplasm, at least in part, during preparation of BC.

It will be noticed that the amounts of calcium (both absolute and relative) increased with increasing boron concentration. In all probability this is indicative of an increase in calcium proteinate—it certainly seems safe to say an increase in cytoplasmic calcium. However BC by no means represents the total cytoplasmic calcium, rather that part of it which was readily extracted due to dilution and pH effects. It almost certainly contained boron and calcium originally associated with the compounds of fraction I ("protein").

In boron-deficient leaves the relatively great amount of boron in BC suggests that cytoplasmic boron is the most important form of this element in the cell. Comparison with the I ("protein") and D ((filtrate from I) values indicates that if the boron is associated with protein, it is easily dissociable and dissolved during the washing process.

The most striking feature was the large increase in cytoplasmic calcium induced by excess boron (a high percentage of the latter also being accommodated in the cytoplasm).

*Fraction D—filtrate from protein precipitation.* A small amount of the boron and calcium of the wall material was presumably extracted during the grinding operation, and appeared in this fraction, together with a certain amount washed out of ruptured chloroplasts. By far the greatest proportion of the boron and calcium in this filtrate, however, was liberated from the compounds of fraction I ("protein") by the trichloracetic acid treatment. It was therefore complementary to BC ("sap-wash") in the sense that it represented the difficultly soluble cytoplasmic boron and calcium. The effects of the various treatments paralleled those for BC, but were less pronounced.

*Fraction FGH—"chloroplasts".* It was previously mentioned that the amounts of chloroplast material prepared in each case did not bear a constant relation to the total chloroplasts of the leaf, because the ease of separation of this fraction varied with the boron content of the plants. Nevertheless, the data are sufficient to indicate that the boron content of chloroplasts is very low, so low that it can be assumed that boron has no function in this part of the cell.

The most reliable figures for the chloroplast fractions are those in section (i) of Table 2, and these suggest that an increase in boron induced an increase in calcium.

*Fraction I.* No great importance can be attributed to this preparation. It might be mentioned here that the amounts of chloroplast protein, and more especially trichloracetic acid-precipitated protein, were exceptionally small in leaves containing large amounts of boron.

*Fractions J and L—"wall" material.* The sum of these two represented the total boron and calcium of the wall which was not easily soluble in water. (This wall material undoubtedly contained also some cytoplasmic substances which were extremely difficult to separate.) Calcium in the wall, in terms of the total calcium, decreased a little with increasing boron concentrations. Whether any significance can be attached to this is doubtful.

It is interesting to notice that both boron and calcium in the wall are very readily soluble in dilute hydrochloric acid. Moreover the percentages, of the total amounts of these elements which are located in the wall material, are surprisingly high, especially when compared with monocotyledonous tissue.

There can be little doubt that some of the wall calcium exists as calcium pectate, whose presence there is fairly well established. Whether pectic acid (and pectinic acids) is sufficient to account for the whole of the calcium in the cell wall, and intercellular substance, is not known. Calcium pectate contains 7·66 p.c. calcium; therefore it would be necessary for normal squash leaves to contain 2·7 gm. calcium pectate, per 100 gm. fresh weight of leaves (dry-weight approximately 10 gm.), for this to be the case[3].

In some plant species, much of the insoluble calcium could be present as calcium oxalate, which would be washed out slowly by cold water, and much more rapidly by hot, if it were actually in the wall structure. On the other hand, calcium oxalate in the familiar form of crystals in the protoplasm, or on the wall surface, would probably appear in fractions other than J and L ("wall" material). Insufficient is known, about other carbohydrate constituents of the wall, to decide whether they are capable of immobilizing any appreciable amount of calcium. Elucidation of the composition of protopectin would clarify this problem a great deal.

The small amount of insoluble boron and calcium in corn, the much greater values in squash, the comparative difficulty in demonstrating boron deficiency in monocotyledons, together suggest that the metabolism of boron and calcium, and some wall substance such as protopectin or pectate, are closely interrelated. Determination of the constitution of the boron compound in the cell wall would be a big step towards the final solution of the functions of this element in higher plants. Presumably the compound with which boron is associated is one which does not occur in the intercellular material of animals, thus accounting for the rapid elimination of boron from the animal, and its very low normal concentration (Bertrand, 1938). It is not impossible that there are complexes of boric acid, and of calcium, with galacturonic acid, galactose, and arabinose units of compound polymers (such as protopectin is likely to be) which are part of the solid structure of the walls of higher plants.

In general, the treatments necessary to separate constituents of cell walls are far too drastic to permit isolation of the unhydrolyzed borate complex. The only promise, then, lies in the testing of complex-forming ability of boric acid with products isolated from cell walls. Our efforts thus far with pectic compounds have been unsuccessful.

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[3] It has since been shown that all the calcium, in the cell walls of the squash leaves, was not present as calcium pectate or pectinates. 150 gm. of fresh normal squash leaves were treated as previously described, except that the acid extraction was omitted. The residue of wall material was rewashed twice with distilled water, by grinding in a Blendor, followed by squeezing through washed muslin. Aliquots of the residue were suspended in water. Surprisingly, the resulting mixture had a pH of 9·0, whereas the enzyme we wished to employ had an optimum pH of 4 to 5. Therefore it was necessary to add an equal amount of dilute hydrochloric acid to each aliquot, to attain a pH of 4·5. A small amount of a fungal enzyme (which was known to attack all pectic substances, yielding galacturonic acid) was added to some of the aliquots, and to others sufficient hydrochloric acid to maintain a pH of 2·2. All were incubated at 37° C. for 12 hours, followed by squeezing through muslin, and determination of calcium in the filtrates, with the following mean results:

| pH. | Enzyme. | Mg. Ca per 150 gm. of leaves. |
|---|---|---|
| 2·2 | absent | 200 |
| 4·5 | present | 104 |
| 4·5 | absent | 72 |

The implication in these figures is that 16 p.c. of the wall calcium could have existed in combination with a pectic substance.

SUMMARY.

In the cells of squash leaves, approximately 50 p.c. of the boron and 70 p.c. of the calcium are immobilized in the cell wall or intercellular substance. The two elements are readily extracted from the wall material by dilute acid, but not by dilute alkali. Comparison of the boron distribution in cells of deficient, normal, and high-boron leaves, suggests that this element is of importance in the cytoplasm and wall, but not in the chloroplasts or vacuole.

A positive correlation exists between boron and soluble calcium in squash plants, but the relationship is not as marked as in the corn plants of Marsh and Shive (1941). Increasing amounts of boron induce appreciable increases in cytoplasmic calcium. This calcium is believed to be associated with proteins.

Radioactive strontium can be used as a qualitative tracer for calcium in the cell, but the representation is not quantitative. In the leaves of normal tomato plants, strontium does not attain the concentrations in chloroplasts and vacuoles that is expected by analogy to calcium. Boron deficiency results in an increase of soluble strontium in tomato leaves.

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# THE EFFECT OF TEMPERATURE AND DRYNESS ON THE SURVIVAL OF THE EGGS OF *CALANDRA ORYZAE* L. (SMALL STRAIN) AND *RHIZOPERTHA DOMINICA* FAB. (COLEOPTERA)

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*(Accepted for publication 3rd August, 1944.)*

When estimating the effect of atmospheric moisture on insects, earlier workers used relative humidity as an index. More recent investigators have found that the conception of "saturation deficit" is more appropriate. Dalton's law implies that atmospheric saturation deficit is proportional to the rate of evaporation from a free water surface. However, experiments have shown that the rate of loss of water from an insect's body is not always proportional to the saturation deficit of the atmosphere. The relationship holds at particular temperatures for some adult insects, but it is not independent of temperature. (Johnson, 1940; Ramsay, 1935:) Experiments by the present writer with the eggs of *C. oryzae* and *R. dominica* showed that there is an interaction between the influence of these factors on the rate of development of the eggs (Birch, 1944). Evans (1934) has shown that when the atmospheric humidities at different temperatures are such that the same amount of water would be evaporated from a free water surface, the mortality of *Lucilia* eggs is higher the higher the temperature. The data of Maercks (1933) for *Habrobracon* eggs also suggests this. These and other examples show that it is impossible to abstract temperature or moisture to consider them as independent factors. In this paper they have been considered together as interacting factors in their influence on the mortality of eggs of *C. oryzae* and *R. dominica*.

## MATERIALS AND METHOD.

Eggs were obtained from laboratory cultures of adults one to five weeks old. The cultures of *C. oryzae* were kept at 26° C. and those of *R. dominica* at 34° C. The eggs of *C. oryzae* were obtained by a method similar to that used by Anderson (1938). The method depends upon the preference the insects have for laying eggs in grain which has not been contaminated by insect activity. When cultures of insects were "conditioned" by increasing the density of the insects, eggs were laid for preference in uncontaminated grain. Wire trays of fresh grain were placed on the surface of such cultures. After 14 hours the trays were removed and eggs dissected from the grain. A small section of endosperm was left attached to the anterior end of each egg. The eggs were arranged on pieces of blotting paper marked in squares and set in the bottom of petri dishes. Eggs of *R. dominica* were obtained by burying thin cork discs mounted on pins, in cultures of adults one to five weeks old. After 14 hours the discs were removed and examined for eggs; damaged eggs were discarded. The remaining eggs were incubated *in situ* on the cork discs. Three replicates of 25 eggs were used for each treatment for *C. oryzae* and five replicates for *R. dominica*. Temperature was controlled to $\pm 0{\cdot}1°$ C. Humidity was controlled with sulphuric acid solutions in Fowler jars.

For the purpose of these experiments, mortality was defined as the percentage of eggs which failed to hatch. At the extremes of the temperature range, some eggs failed to hatch though they had completed embryonic development.

## TEMPERATURES AT WHICH *C. ORYZAE* AND *R. DOMINICA* CANNOT HATCH.

At favourable relative humidities there is a medial range of temperatures over which the mortality of eggs of both these species is low: for the eggs of *C. oryzae* this range is 15° C. to 33·5° C. and for *R. dominica* the range is 18° C. to 38° C. (Author's unpublished data). Beyond

this range of temperatures the mortality rate increases at all levels of moisture. This is shown in Table 1. At all temperatures shown in this Table the eggs were at a relative humidity of 85 p.c.

TABLE 1.

*Showing the mortality of eggs at high temperatures.*

| *Calandra oryzae.* | | *Rhizopertha dominica.* | |
|---|---|---|---|
| Temp. °C. | P.c. of eggs which failed to hatch. | Temp. °C. | P.c. of eggs which failed to hatch. |
| 33·5 | 15 | 38·4 | 4 |
| 34·0 | 20 | 38·6 | 19 |
| 34·4 | 70 | 38·8 | 91 |
| 34·6 | 100[1] | 39·0 | 91 |
| 34·8 | 100 | 40·0 | 100 |

All differences are significant at P = 0·01.

At 34·6° C. none of the eggs of *C. oryzae* hatched, whereas at 34·0° C. 80 p.c. hatched. At 13° C. one p.c. of the eggs of *C. oryzae* hatched. Practically all the remaining eggs contained fully developed embryos, but they did not hatch at this temperature. The one p.c. of eggs which hatched, did so after 33 days at 13° C. At 40° C. none of the eggs of *R. dominica* hatched, but at 38·6° C. 81 p.c. hatched.

At the temperatures shown in Table 1 nearly all the eggs which failed to hatch completed embryonic development. As the embryos of *C. oryzae* and *R. dominica* approach maturity, the cuticle of the egg disappears (presumably due to the action of hatching enzymes). The remaining embryonic membranes are then ruptured. In the eggs of *R. dominica* this is done by means of a hatching spine situated on the tail segment of the embryo. Prior to hatching, the tail segments are flexed so that the spine can be seen through the chorion in about the centre of the egg. With the unflexing of the tail the membranes are ruptured in the mid-ventral line from the centre of the egg to the posterior pole. At the temperatures shown in Table 1 the cuticle had disappeared in the normal way, but hatching was prevented by the failure of the tail to unflex. The embryos of *C. oryzae* do not possess hatching spines—the rupturing of the embryonic membranes appears to depend on rhythmic contractions in the length of the larva.

The mortality rates shown in Table 1 were plotted against temperature. The resulting curves did not conform to normal dosage mortality curves. Most of the eggs were susceptible to a narrow range of temperature. Between 34·0° C. and 34·6° C. the mortality of eggs of *C. oryzae* increased from 20 p.c. to 100 p.c. Between 38·6° C. and 39·0° C. the mortality of eggs of *R. dominica* increased from 19 p.c. to 39 p.c. This shows that there is a skew distribution of the frequency curve of the susceptibility of eggs to the lethal effect of high temperature.

## THE EFFECT OF DRYNESS ON HATCHING OF EGGS, OVER THE RANGE OF TEMPERATURES AT WHICH EGGS HATCH WHEN MOISTURE IS NOT LIMITING.

When eggs were incubated at a favourable relative humidity within the medial range of temperatures already referred to, the mortality rate was low. The mortality rate of eggs which were exposed to various humidities within this range of temperatures is shown in Table 2.

TABLE 2.

*Showing the mortality of eggs at different combinations of temperatures and relative humidity.*

| *Calandra oryzae.* | | | | | |
|---|---|---|---|---|---|
| | Relative humidity. | | | | |
| Temp. | 20 | 27 | 35 | 50 | 90 |
| 15·0 | 100 | 100 | 92 | 29 | 26 |
| 18·3 | 100 | 100 | 31 | 15 | 10 |
| 23·0 | 100 | 80 | 27 | 19 | 9 |
| 25·5 | 100 | 97 | 37 | 20 | 6 |
| 29·0 | 100 | 100 | 68 | 22 | 2 |
| 32·0 | 100 | 100 | 100 | 41 | 13 |

At P = ·01 significant difference = 10.

| *Rhizopertha dominica.* | | | | | | |
|---|---|---|---|---|---|---|
| | Relative humidity. | | | | | |
| Temp. | 0 | 15·5 | 21 | 33·5 | 51·5 | 90 |
| 18·3 | 100 | 100 | 100 | 100 | 87 | 8 |
| 30·0 | 56 | 23 | 10 | 0 | 0 | 0 |
| 34·0 | 73 | 42 | — | 20 | 2 | 0 |
| 38·2 | 100 | 100 | 96 | 34 | 29 | 0 |

At P = ·01 significant difference = 17.

[1] 30 p.c. of the larvae inside the eggs ruptured the chorion but did not emerge.

The data of Table 2 have been analysed to determine the relation between temperature, dryness and mortality. The relation between saturation deficit and mortality for any one temperature was curvilinear. For any particular saturation deficit there was a higher mortality at the lower temperatures. This was to be expected since the time of exposure was longer at the lower temperatures because development was slower. The time factor was taken into account when mortality was plotted against the value of saturation deficit (s.d.) × time (t), where time is the length of development at a relative humidity where hatching occurs. The relation between mortality and s.d. × t. was not linear and it was not independent of temperature (Fig. 1). For any particular value of s.d. × t. mortality was higher the higher the temperature.

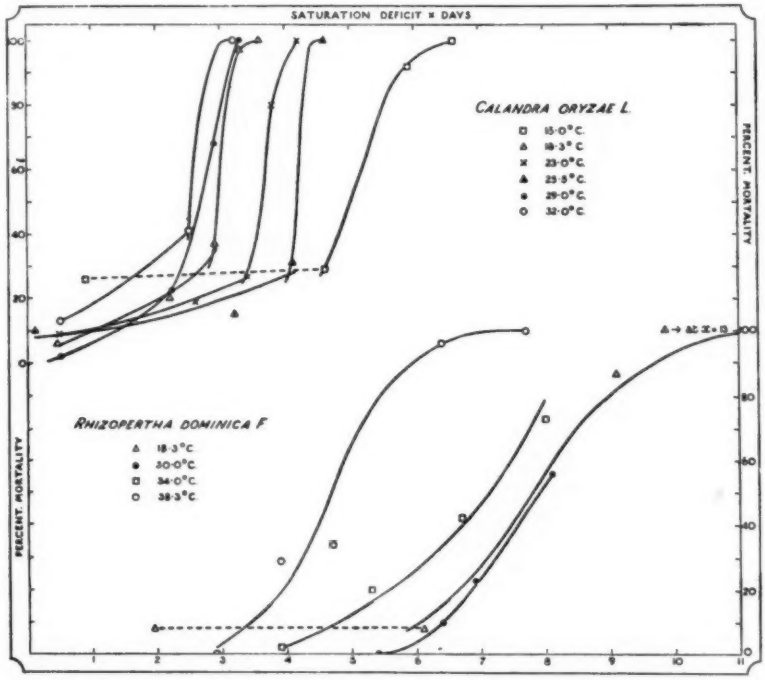


**Fig. 1.** Showing the mortality of eggs at different conditions of temperature and dryness.

Mellanby (1935) postulated that if the rate of loss of water from eggs was proportional to saturation deficit, then mortality due to desiccation would be proportional to the product of saturation deficit × time of desiccation (i.e. linear). It does not, however, follow that if the amount of water evaporated is proportional to saturation deficit × time, that the mortality rate will also be proportional to this product. It is more probable that the relationship would be sigmoidal like other "dosage-mortality" curves.

The curves in Fig. 1 have been interpreted as "dosage-mortality" curves. When probits for mortality were plotted against s.d. × t. on the abscissa, the points for any one temperature were in most cases disposed about two straight lines. In some cases one straight line could be drawn through all the points. The points fitted the straight line at the 1 p.c. level of probability. The fact that two lines gave a better fit than one indicates the skewness of the frequency curve for minimum lethal dosage, where dosage is the product s.d. × t. This is evident from the flat nature of the curves in Fig. 1 over the first half of their abscissal range. In other words, the influence of dryness on mortality is not severe until a critical value of dryness is reached, and the range beyond this value at which any survival occurs is less than the range below it. The curves drawn in Fig. 1 are the converted probit curves.

In Fig. 2 the values of s.d. × t. corresponding to a mortality of 50 p.c. have been plotted against temperature. This shows the dependence of mortality at a particular evaporating power of the air on temperature. The values were calculated from the probit curves; 50 p.c. mortality rate was chosen because the values of s.d. × t. to produce this mortality rate were determined with the greatest accuracy. The variance of each value has been calculated (Bliss, 1938). These variances are shown in Fig. 2 as vertical lines corresponding to odds at $P = 0{\cdot}05$ (i.e. each line is drawn about the observed value as ± standard deviation × 1·96). In units of s.d. × t. these values range from ± 0·07 to 0·16 for *C. oryzae* and ± 0·15 to ± 0·27 for *R. dominica*. At $P = 0{\cdot}5$ all the points in Fig. 2 are significantly different from each other excepting the values at 29° C. and 32° C. for *C. oryzae*.

The following curves were fitted to the points in Fig. 2 to describe the relationship of temperature and s.d. × t. for a mortality rate of 50 p.c.

*C. oryzae* $y = 9{\cdot}865 - 0{\cdot}414\,x + 0{\cdot}0057\,x^2$

[2] *R. dominica* $y = 19{\cdot}787 - 0{\cdot}391\,x$

where y = saturation deficit × time (in days)

x = temperature °C.

The coefficients for linear regression for *C. oryzae* and *R. dominica* are − 0·145 and − 0·391 respectively. This means that for a decrease in temperature of 1° C. the eggs of *R. dominica* can withstand an increase in s.d. × t. three times as great as that for *C. oryzae* for the some mortality. (The figure for *C. oryzae* is less accurate than that for *R. dominica*, as it averages the slope of a curvilinear relation).

The curve for eggs of *R. dominica* in Fig. 2 covers a higher range of values on the y axis than does the curve for *C. oryzae*.. This is to say, at any temperature the eggs of *R. dominica* can withstand much greater desiccation. The eggs of *R. dominica* are laid outside the grain and are normally subjected to more severe desiccating influences than the eggs of *C. oryzae*, which are buried in the grain. The eggs of the pea weevil which are laid outside the grain are similarly resistant to desiccation (Menusan, 1934).

The regression of (saturation deficit × time) on temperature for a particular mortality rate, shows that there is no simple relationship between mortality and the evaporating power of the air. The value of the regression, moreover, is different for different mortality rates. Two factors may be operating to give this regression—the rate of loss of water from eggs at a particular saturation deficit may not be independent of temperature, or there is an interaction between temperature and moisture loss in their influence on mortality rate. It is evident that saturation deficit cannot be considered alone as a factor determining mortality.

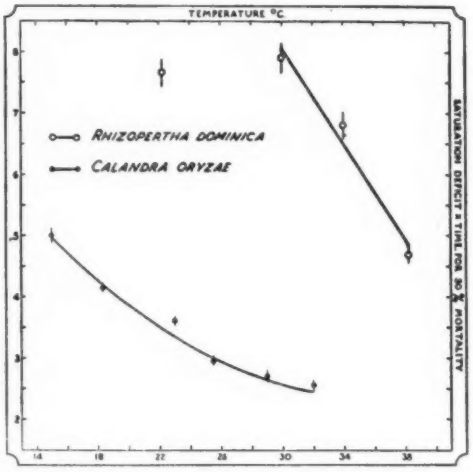


**Fig. 2.** Showing the relation between temperature and the evaporating power of the atmosphere (saturation deficit × time) to give a mortality rate of 50 p.c. The calculated curve rate for *R. dominica* is

$$y = 19{\cdot}787 + 0{\cdot}391\,x,$$

that for *C. oryzae* is

$$y = 9{\cdot}865 - 0{\cdot}414\,x + 0{\cdot}0057\,x^2.$$

The vertical lines show the value of significant differences for each point at $P = 0{\cdot}05$.

## SUMMARY.

No eggs of *C. oryzae* hatched below 13° C. or above 34·6° C.
No eggs of *R. dominica* hatched above 40° C.

[2] The figure for 18·3° C. has not been included in this curve. There is evidence that 18·3° C. is about the lowest temperature at which hatching will occur, and that low temperature is having a direct effect on mortality at this temperature.

The mortality rate of eggs at any one temperature was not proportional to saturation deficit, nor to the product of saturation deficit x time. The mortality curves at any one temperature were interpreted as "dosage-mortality" curves by the method of probits. For most temperatures two probit lines were fitted.

There was no simple relation between evaporating power of the air and mortality. Differences in temperature as small as 2° C. gave significant differences in mortality when the evaporating power of the air (expressed as a product of s.d. × time) was kept constant. The higher the temperature the higher was the mortality for a particular value of s.d. × t.

The relation between temperature (x) and evaporating power (s.d. × t.) to give 50 p.c. mortality rate for *C. oryzae* eggs was given by the parabolic formula

$$\text{s.d.} \times \text{t.} = 8{\cdot}9865 - 0{\cdot}414\,x + 0{\cdot}0057\,x^2$$

For eggs of *R. dominica* the relation was linear.

$$\text{s.d.} \times \text{t.} = 19{\cdot}787 - 0{\cdot}391\,x$$

For a decrease in temperature of one degree C. the eggs of *R. dominica* can withstand an increase in s.d. × t. three times as great as that for *C. oryzae* for the same mortality.

At any particular temperature the eggs of *R. dominica* were able to withstand much greater desiccation than those of *C. oryzae*.

***Acknowledgment.*** Grateful acknowledgment is made to Professor J. Davidson and Mr. H. G. Andrewartha for their helpful advice and criticism.

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# TWO STRAINS OF *CALANDRA ORYZAE* L. (COLEOPTERA)

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*(Accepted for publication, 3rd August, 1944.)*

During an examination by Mr. F. J. Gay and the writer, of adults of *Calandra oryzae* reared at the Waite Institute, and of adults of this species reared by Mr. Gay at the Division of Economic Entomology in Canberra, it was observed that the latter adults were larger than those reared at the Waite Institute. The Canberra cultures were started with individuals taken from infested maize in store in Victoria · the Waite Institute cultures were started with individuals taken from infested wheat in store in South Australia.

The information given in this paper shows that the cultures consist of two distinct forms of the species which can be conveniently referred to as "small strain" and "large strain."[1] Both forms possess markings on the elytra which are characteristic for the species. In a preliminary examination of stored wheat in Victoria, South Australia, New South Wales, and Western Australia, only the small strain has been found. An examination of the species occurring in stored grain in Queensland has not yet been made.

The discovery of strains of insects which are morphologically similar but which differ in physiological characteristics is becoming an increasingly familiar experience to biologists. A number of these strains can be separated on differences in response to temperature and moisture, e.g. the two races of *Trichogramma minutum* (Lund, 1934), the two races of *Drosophila pseudoobscura* (Poulson, 1934), and the two races of *Austroicetes cruciata* (Andrewartha, 1944). This paper deals with interbreeding experiments and differences in the influence of temperature and moisture in the two strains of *C. oryzae*.[2]

## DIFFERENCES IN SIZE OF THE TWO STRAINS.

The length of the pronotum, the total length and maximum width of the body were determined for 50 adults of each sex and race (Table 1). Measurements were made to the nearest one-tenth of a millimeter by means of the stage vernier of a binocular microscope.

TABLE 1.

*Showing the dimensions in millimetres of the two strains.*

| | Large strain. | | | | Small strain. | | | |
|---|---|---|---|---|---|---|---|---|
| | Male. | | Female. | | Male. | | Female. | |
| | Mean. | C.V. | Mean. | C.V. | Mean. | C.V. | Mean. | C.V. |
| Pronotum length | 1·22 | 7·8 | 1·20 | 6·3 | 1·02 | 6·9 | 1·04 | 6·4 |
| Total length | 2·88 | 5·9 | 2·86 | 6·4 | 2·43 | 5·7 | 2·50 | 5·6 |
| Maximum width | 1·12 | 7·0 | 1·12 | 6·3 | 0·93 | 6·5 | 0·96 | 6·2 |

At $P = 0{\cdot}01$ significant differences of $\log (x + 1) = 0{\cdot}03$ where $x$ = mean values shown in Table.

By using the transformation of $\log (x + 1)$ it has been possible to give one figure for significant differences between any of the parts measured. All parts of the large strain are significantly larger than those of the corresponding sex of the small strain. There is no significant difference in the coefficients of variability for the two strains.[3] The coefficient of

[1] It is reported that Dr. O. W. Richards has recorded the presence of two size forms of *C. oryzae* in Britain (Howe, 1943).

[2] The two strains were bred through several generations at 26° C. in wheat of 14 p.c. moisture content before the comparative experiments were made.

[3] Significant differences between the coefficients of variability have been determined from Tables giving their standard errors (Brown, 1934).

variability is the most suitable index to use in this case as it expresses variation as a percentage of the mean. The fact that these values are similar for the two strains, shows that the variances of the strains are different. These differences are significant. It is probably because of this difference that a subjective judgment would suggest that the large strain is more variable than the small strain.

## INTER-STRAIN CROSSES.

A series of six different crosses was made with the two strains. Adults which had not previously mated were obtained by isolating, in tubes, single grains which contained an insect in the pupal or late larval stage. When the adults had emerged from the grains, the sex was determined.[4] Pairs appropriate to the particular cross were then confined in tubes containing 40 grains of wheat. After three weeks at 30° C. in an atmosphere of 75 p.c. relative humidity, the pairs of insects were removed. At the end of six weeks the tubes were examined for progeny which had emerged.

The prefixes L. and S. in the six crosses listed below stand for large and small individuals of the large strain. This distinction was made because of the possibility of the small strain being able to cross with the small individuals of the large strain, but not with the large ones. Individuals which measured 2·7 mm. or less in total length were designated as small (S.); 2·7 mm. was the maximum length of any individuals of the small strain; 18 p.c. of the males and 30 p.c. of the females of the large strain measured 2·7 or less. It was not practicable to choose a smaller figure, only 2 p.c. of the large strain, for example, were the same length or less, than the mean length of the small strain. Individuals of the large strain which measured 2·8 mm. or more in length were designated as large (L.).

The following six crosses were made. Each one of these was replicated ten times:

(A) L. Large ♂ x Small ♀
(B) L. Large ♀ x Small ♂
(C) S. Large ♂ x Small ♀
(D) S. Large ♀ x Small ♂
(E) S. Large ♂ x L. Large ♀
(F) S. Large ♀ x L. Large ♂

The intra-strain crosses (E and F) proved to be fertile: at the end of six weeks each pair had given rise to from 20 to 40 adult progeny. All inter-strain crosses (A, B, C and D) were sterile, except for the production of two males from one replicate of cross B, and one male and one female from two replicates in cross C. In the crosses A, B, C and D some eggs were laid. About 10 p.c. of these eggs had developed mature embryos, but none had hatched.

## THE INFLUENCE OF TEMPERATURE ON THE DEVELOPMENT OF THE TWO STRAINS.

The following experiment was carried out to determine

(1) if the temperature at which the two strains developed from egg to adult in the shortest time, was same for the two strains; and

(2) to compare the length of the developmental period for the two strains at the same constant temperatures.

Replicates of 150 grains, each containing one egg, were incubated at 75 p.c. R.H. at 25·5° C., 27·5° C. and 30° C.[5] The grains were examined twice per day as soon as adults began to emerge. The mean time taken for the development from the laying of the egg to the emergence of the adult from the grain[6] is shown in Table 2.

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[4] Sexes were determined on the size of the rostrum—the rostrum is shorter and thicker in the male. With practice, it was found that a correct decision could be made in 95 p.c. of the cases. All insects were dissected at the end of the experiment to verify the sex.

[5] The method adopted for finding the eggs in the grain was similar in principle to the "candling" of hens' eggs. The stage of a binocular microscope was blacked out except for a point source of light which shone through a central small hole. Each grain was examined over this beam of light. Eggs buried in the endosperm are more transparent than the grain and show up as translucent spots. By turning over the grain with a needle and puncturing all but one egg in each grain, it was possible to obtain a large number of grains each containing one egg.

[6] In a preliminary experiment it was found that the adults stayed within the grain before boring their way out, for the same time (3·5 days) at the three temperatures of this experiment. These temperatures are in the upper part of the range of temperature over which development from egg to adult can take place. The complete range for the small strain is 18° C. to 33·5° C. (unpublished data); the complete range for the large strain has not been determined.

At each temperature in Table 2 the small strain developed in a shorter time than the large strain. The coefficients of variability of the large strain were significantly greater at each temperature. At 25·5° C. the coefficient was twice that of the small strain. This marked difference in variability in the length of development is illustrated in Fig. 1. It shows the frequency curve for the length of development of 150 individuals at 30° C. This difference in variability cannot be wholly accounted for by the larger "variance" in size of the large strain, because the coefficient of variability for size was not significantly different for the two strains.

The mean of the reciprocals of time to complete development have been plotted against temperature (Fig. 2). The resulting curves are part of the "temperature-velocity" curves for these insects. The rate of increase in rate of development of the small strain per degree rise in temperature was the same between 25·5° C. and 27·5° C., and 27·5° C. and 30·0° C., i.e. the temperature velocity curve is linear within this range. The rate of increase in rate of development per degree rise in temperature for the large strain was less between 27·5° C. and 30·0° C. than between 25·5° C. and 27·5° C., i.e. the temperature velocity curve is curvilinear within this range.

To determine if this difference between the two strains was significant, it was necessary to transform to the log scale. It was not possible to determine significant differences between rates of increase directly. It can be done by comparing differences over equal temperature intervals. The temperature intervals, however, between 25·5°–27·5° C. and 27·5°–30·0° C. are not the same. To overcome this difficulty a value for 29·5° C. has been calculated by linear extrapolation for each replicate. This calculation has also made the appropriate adjustment to the variance, since the variance is proportional to the mean in this experiment.

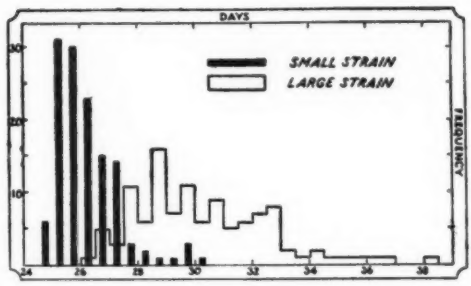


**Fig. 1.** Showing the difference in variability of the two strains in the time taken to develop from egg to adult at 30° C.

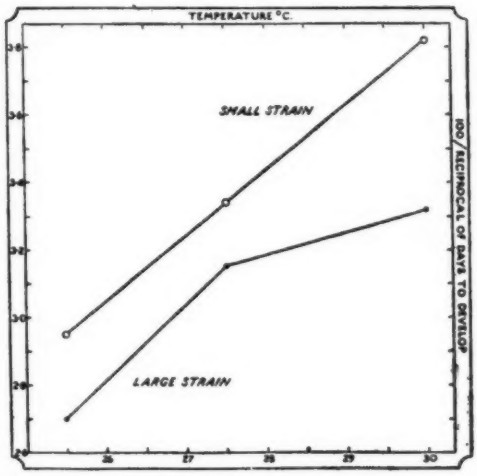


**Fig. 2.** Showing the difference in the rate of development of the two strains at 25·5° C., 27·5° C. and 30·0° C.

TABLE 2.

*Showing days necessary for the development of the two strains, from the laying of the egg to the emergence of the adult from the grain, at three constant temperatures (C.V. = coefficient of variability).*

| | Small strain. | | Large strain. | |
|---|---|---|---|---|
| Temp. °C. | Mean. | C.V. | Mean. | C.V. |
| 25·5 | 33·96 | 3·8 | 37·30 | 8·4 |
| 27·5 | 29·98 | 4·4 | 31·90 | 6·4 |
| 30·0 | 26·20 | 4·1 | 30·30 | 7·8 |

**At P = 0·01 all differences between means are significant.**

The difference between the logs of the geometric means is:

| | Temperature difference. | | Difference | Significant difference |
|---|---|---|---|---|
| | 25·5°–27·5° C. | 27·5°–29·5° C. | in logs. | at P = 0·01. |
| Small strain | 0·054 | 0·047 | 0·007 | 0·012 |
| Large strain | 0·067 | 0·018 | 0·048 | 0·013 |

At P = 0·01 the difference for the large strain is significant. This shows that the rate of increase in rate of development per degree rise in temperature between 27·5° C. and 30·0° C. is significantly less than the rate between 25·5° C. and 27·5° C. In other words, the rate of increase in rate of development falls with temperature increase between 25·5° C. and 30·0° C. for the large strain, but it remains the same for the small strain.

A striking difference between the two strains was the fact that the larva of the large strain bores a hole to the outside of the grain, and through this large quantities of frass material are passed—a habit which resembles that of the larvae of *Rhizopertha dominica*. The larvae of the small strain rarely make holes to the outside of the grain, although the phenomenon has been observed when there was more than one larva in the grain.

## THE INFLUENCE OF MOISTURE ON THE OVIPOSITION OF THE TWO STRAINS.

A comparison was made of the total eggs laid by the two strains at different temperatures in wheat of different moisture contents. The two factors to be determined were

(1) whether the potentiality to lay eggs in grain of low moisture content was different for the two strains, and

(2) if there was a statistical interaction between strain and moisture content of wheat, i.e. to determine if a given reduction in moisture content of wheat produced in both strains the same percentage reduction in number of eggs laid.

The total number of eggs laid was determined at 25·5°C. and 30·0° C., in wheats of 9 p.c., 10 p.c. and 11 p.c. moisture content respectively. There were three replicates for each treatment. For each replicate five pairs of weevils were enclosed in a glass tube of 1" diameter which was filled with 100 grains of wheat. The ends of the tube were closed with phosphor bronze gauze. These oviposition cages were kept at the desired humidity in Fowler jars over sulphuric acid solutions of the appropriate concentration. The grain was changed every ten days and the eggs counted by the method already described (footnote [5], page 272).

### TABLE 3.

*Showing the mean number of eggs laid by the two strains in wheat of low moisture content (for 5 females).*

| Temp. | 25·5° C. | | | 30·0° C. | | |
|---|---|---|---|---|---|---|
| P.c. moisture content of wheat | 9 | 10 | 11 | 9 | 10 | 11 |
| Small strain | 0 | 55 | 359 | 0 | 62 | 331 |
| Large strain | 0 | 25 | 405 | 0 | 14 | 290 |

The results of the experiment could not be analysed together in one Table of variance owing to the different variances under the different treatments, and the inability of any transformation to equalize them. Treatments have therefore been compared in pairs. At both temperatures the large strain laid significantly fewer eggs (P = 0·01) in wheat of 10 p.c. moisture content than the small strain. The numbers of eggs laid at both temperatures by the small strain in wheat of 11 p.c. moisture content were not significantly different, but with the large strain fewer eggs were laid at 30·0° C. than at 25·5° C. There is thus an interaction between temperature, moisture and strain. There was no significant difference in the number of eggs laid by the two strains at 25·5° C. in wheat of 11 p.c. moisture content. As the temperature was raised above this, or the moisture content reduced, the number of eggs laid by the large strain was less than the number laid by the small strain.

## SUMMARY.

This paper records two strains of *Calandra oryzae L.* present in Australia. The strains differ significantly in the length of the pronotum, the length and maximum width of the body. The large strain develops at a slower rate at constant temperatures, and the rate of increase in rate of development falls with temperature increase between 25·5° C. and 30·0° C. for the large strain, but remains constant

for the small strain. There is no significant difference in the variability in size of the body of the two strains, but the variability in the time required for the development of the large strain is significantly greater than that of the small strain. The temperature for maximum oviposition in wheat of 11 p.c. moisture content is lower for the large stain, and fewer eggs are laid by the large strain in wheat of 10 p.c. moisture content.

The two strains were inter sterile. This was not due simply to a physical difference in size, as small individuals of the large strain did not cross with the small strain. Some eggs were laid as a result of these inter-strain crosses, and about 10 p.c. developed to mature embryos, but none hatched. This has also been observed in some of the inter-race crosses of *Anopheles maculipennis* (Thorpe, 1940).

On the basis of Dobzhansky's (1941, p. 373) criteria for species differences, it would appear that the two forms of C. *oryzae* could be classed as different species.

*Acknowledgments.* Grateful acknowledgment is made to Professor J. Davidson and Mr. H. G. Andrewartha for their helpful advice and criticism. Acknowledgment is also made of the assistance of Mr. R. Strahan in some of the experimental work; he also kindly collected and examined samples of infested wheat from storages in Western Australia.

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# AN IMPROVED METHOD FOR DETERMINING THE INFLUENCE OF TEMPERATURE ON THE RATE OF DEVELOPMENT OF INSECT EGGS. (USING EGGS OF THE SMALL STRAIN OF *CALANDRA ORYZAE* L. (COLEOPTERA)

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*(Accepted for publication 25th September, 1944.)*

In the determination of the time taken for the development of insects' eggs in the field, it is necessary to construct the so-called temperature velocity curve. The reciprocals of the time taken for the eggs to develop at constant temperatures are plotted against temperature. The trend of these points is sigmoid (Davidson, 1942). It has been shown (Andrewartha, 1944) that small errors made in the computation of the temperature velocity curve may introduce considerable errors in the determination of the time necessary for the eggs to develop in the field. For this reason it is desirable that the appropriate curve should be established with precision.

Experiments have been carried out to determine the temperature velocity curve for the eggs of the small strain of *Calandra oryzae* L. Several new conceptions have been developed to reduce the errors associated with the technique of this type of experiment.

## MATERIALS AND EQUIPMENT.

The eggs were laid in wheat of 14 p.c. moisture content at 26° C. by insects 1–5 weeks old. Stock cultures of the insects were kept at 26° C. They had been started twelve months previously from some hundreds of individuals taken from a bag stack.[2] Eggs were obtained by a method previously described (Birch, 1944). For each treatment two replicates of 100 eggs were used. The eggs were then arranged on pieces of blotting paper marked in squares and set in the bottom of petri dishes.

Atmospheric humidity was controlled in Fowler jars, in which the petri dishes were placed, by means of sulphuric acid solutions. Each jar was provided with an internal centrifugal fan enclosed in a tin attached to the lid (Fig. 2). The fan was operated from an external pulley wheel. Air drawn in at the central opening of the tin was forced through a lateral tube over the surface of the acid. A circulation of air could be maintained within the jar in this way. It was thus possible to restore the relative humidity soon after the jar had been opened. Four such jars formed a battery driven from a central spindle. This was in turn geared to a fan shaft inside the incubator (Fig. 1). The temperature of each incubator was controlled within $\pm 0{\cdot}1°$ C.

## THEORY AND METHOD OF EXPERIMENT.

The eggs of most insects develop in nature in an atmosphere which is not saturated with moisture. They may lose water in an unsaturated atmosphere, but the loss is so slow that normally the eggs hatch long before a moisture content in equilibrium with the surrounding atmosphere is

[1] This work was carried out with the aid of a grant from the Federal Research Grant to the University of Adelaide.

[2] Certain workers who have experimented with insects of stored products have made special efforts to use insects which were descended from a single pair of individuals or a small number of individuals. Such insects are then said to be inbred stocks. Inbreeding, however, necessitates a series of brother-sister crosses in several successive generations. The insects derived from the indiscriminate crossing of the progeny of a single pair are likely to be almost as variable in genetic constitution as those derived from a number of pairs (Walter, 1931, *et al.*).

attained. For this reason a constant relative humidity at different temperatures does not give constant moisture conditions at these temperatures.

The rate of development of eggs at different temperatures is more likely to be affected by the total water lost during incubation than by the rate of loss of water. This is because the total water lost takes into account both the rate of loss and the duration of exposure. In determining the influence of temperature on rate of development, the influence of moisture is therefore most likely to be maintained constant throughout the range of temperature, when the conditions are such that the eggs lose the same amount of water during their incubation. The following experiment was designed to test this hypothesis. It was necessary to determine whether there was an interaction between temperature and moisture, moisture being defined as the total evaporating power of the air during the incubation period of the egg (i.e. as the product of saturation deficit × time).

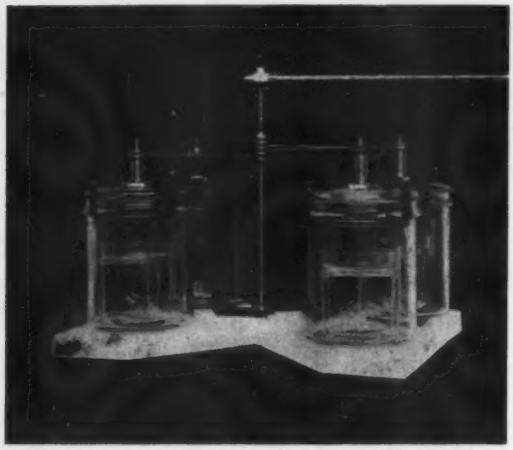


**Fig. 1.** Showing battery of four Fowler jars with internal fans driven from a central spindle. The battery is placed in a constant temperature cabinet. The spindle is driven from the fan shaft in the cabinet.

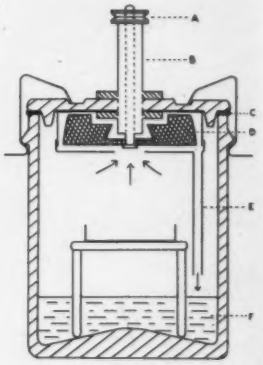



**Fig. 2.** Showing a diagrammatic cross-section of an individual jar:

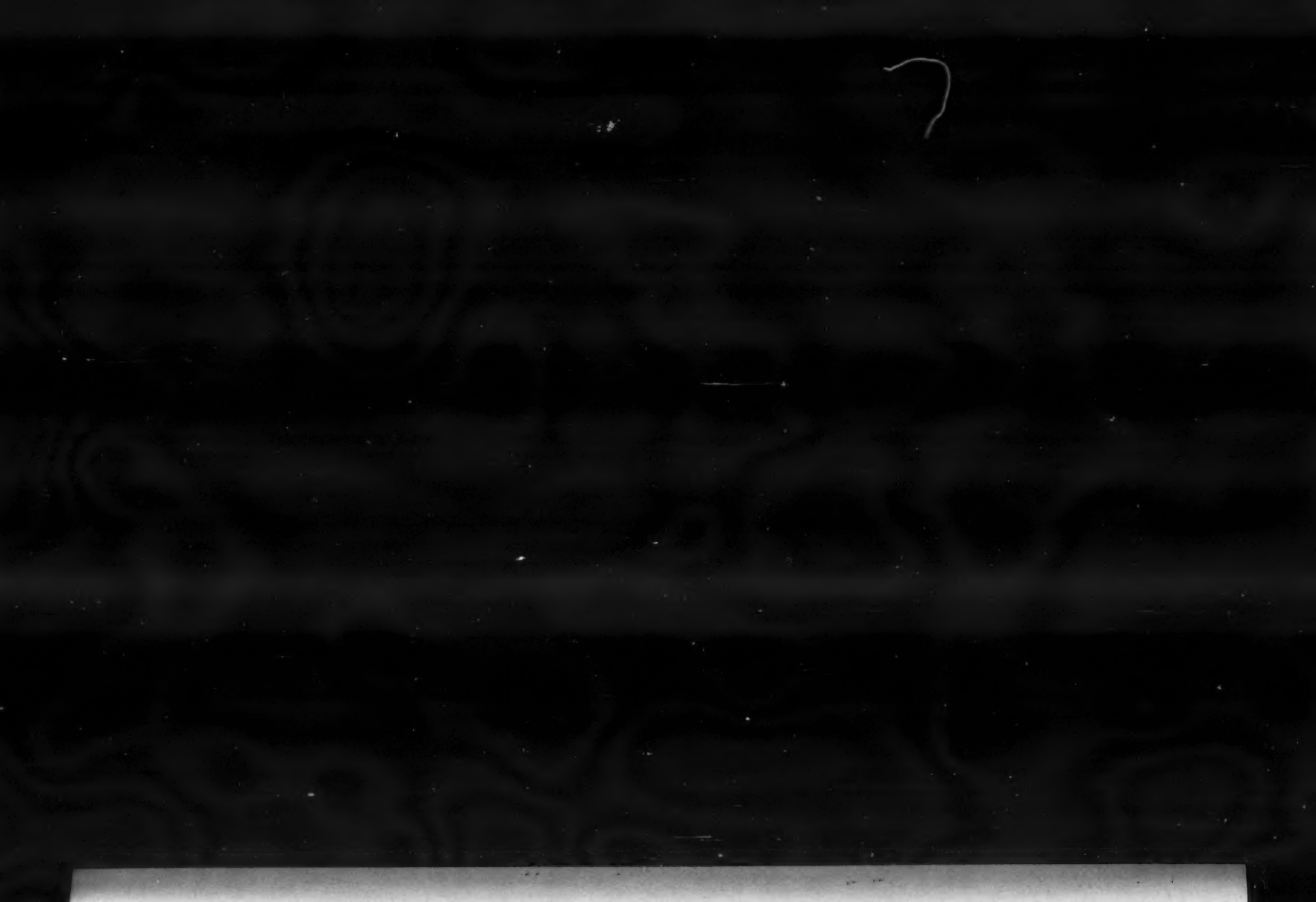
A. Pulley wheel
B. Fan shaft casing.
C. Rubber ring.
D. Fan blade.
E. Air outlet.
F. Sulphuric acid solution.

TABLE 1.

*Showing plan of experiment based on the preliminary estimation of the developmental period at 75 p.c. R.H.*

| Temperature °C. | Approx. days to develop at 75 p.c. R.H. | R.H. at $M_1$ ($M_1 = 0{\cdot}96$). | R.H. at $M_2$ ($M_2 = 0{\cdot}56$). | Operation of fan. Total duration in hours. | Times per day. | Duration per operation, in minutes. |
|---|---|---|---|---|---|---|
| 15·2 | 20·0 | 90 | 95 | 24·0 | 2 | 36 |
| 18·2 | 10·0 | 83 | 91 | 12·0 | 3 | 24 |
| 22·1 | 6·0 | 80 | 88 | 7·2 | 3 | 24 |
| 25·4 | 4·0 | 75 | 85 | 4·8 | 3 | 24 |
| 29·1 | 3·0 | 73 | 84 | 3·6 | 3 | 24 |
| 30·8 | 3·0 | 76 | 85 | 3·6 | 3 | 24 |
| 32·3 | 3·5 | 81 | 89 | 4·1 | 3 | 24 |
| 34·0 | 4·0 | 85 | 91 | 4·8 | 3 | 24 |
| 34·7 | 4·5 | 88 | 93 | 5·4 | 3 | 24 |

The product saturation deficit (s.d.) × time can only be kept approximately constant, since one factor (time) can be determined only approximately until the correct value for the other factor (s.d.) is known. Successive approximations should give results nearer to the true value.



In this case it was considered necessary just to make the first approximation. For this purpose the duration of development of the eggs at different temperatures was determined initially at a constant relative humidity of 75 p.c.[3] (Table 1, column 2). The values of the factor time so obtained were used to calculate the approximate values of relative humidity such that the product of s.d. × time was constant. Two constant values of the product s.d. × time ($M_1$ and $M_2$) were chosen at which to carry out the experiment: $M_1$ (time in days × s.d. in inches of mercury) = 0·96, and $M_2$ = 0·56. These values were chosen on the basis of a preliminary experiment which showed that the zone of equal high survival of eggs lay within the limiting values for s.d. × time of 0·06 and 1·67.

The value of the product s.d. × time in the experiment was only approximately constant over the temperature range used. This was because the initial estimate of the required relative humidities was approximate only. The actual values of the product s.d. × time shown in Table 2 have been calculated from the final estimate of time shown in Table 3. Column four of Table 2 shows the variability in these values at a constant relative humidity of 75 p.c. Comparison with columns two and three shows the improvement gained when the relative humidity is varied. Columns two and three show the accuracy of the first approximation. Further approximation would have reduced the variability of these values, but this was not considered necessary.

TABLE 2.

*Showing the extent to which the value of s.d. × time was maintained constant in the experiment. The values when relative humidity was constant are also shown.*

| | Saturation deficit × time. | | |
|---|---|---|---|
| Temperature °C. | $M_1$. | $M_2$. | 75 p.c. R.H. |
| 15·2 | 0·93 | 0·52 | 2·60 |
| 18·2 | 1·06 | 0·59 | 2·90 |
| 22·1 | 0·99 | 0·58 | 1·24 |
| 25·4 | 1·10 | 0·64 | 1·11 |
| 29·1 | 1·24 | 0·72 | 1·10 |
| 30·8 | 1·12 | 0·62 | 1·16 |
| 32·3 | 0·95 | 0·56 | 1·22 |
| 34·0 | 0·86 | 0·51 | 1·41 |
| 34·7 | 1·01 | 0·62 | 2·02 |
| Mean deviation | 8 p.c. | 8 p.c. | 43 p.c. |
| Range | 44 p.c. | 40 p.c. | 136 p.c. |

The mean deviation and range are expressed as a percentage of the higher values.

In making the final estimate of the factor time, observations during the hatching period of the eggs were made at eight-hour intervals at all temperatures except 15·2° C. At this temperature they were made at 12-hour intervals. It was necessary to open the jars at each observation. The internal fan was operated after the jars had been opened to bring the atmosphere within the jar back into equilibrium with the acid as rapidly as possible. The operation of the fan was arranged so that the air movement factor was constant at all temperatures. This was necessary since the total evaporating power of the air is proportional to the product of saturation deficit and time, only when the air movement factor is constant. The operation of the fan was distributed over the whole period of development and the ratio, total time of operation: total time for complete development, was kept constant for all temperatures. That is, the fan was operated for 1/20th of the developmental period, or 72 minutes per day (Table 1).

The loss of information in the determination of the mean values of time to develop with the group intervals used, i.e. 12 and 8 hours, is shown in column five of Table 3. The percentage loss of information in estimating the mean $= \frac{100a^2}{12\sigma^2}$ where a = group interval and $\sigma^2$ = variance after deducting Sheppard's correction $\left(\frac{a^2}{12}\right)$, (Fisher, 1921).

The figures show that the loss is negligible at 15·2° C and 18·2° C. At the higher temperatures an interval less than eight hours would be desirable though hardly practicable.

[3] A closer first approximation would have been obtained if higher relative humidities had been used at the extremes of the temperature range, i.e. if they had approximated more closely the values shown in column 3 of Table 1. Subsequent work would be planned with this in view.

TABLE 3.

*Showing duration of development and mortality* of eggs at two moisture levels and nine temperatures.*

| Temperature °C. | Duration of development (in hours). $M_1$. | $M_2$. | $M_1$–$M_2$. | P.c. loss of information in estimating mean hours. | P.c. mortality. $M_1$. | $M_2$. |
|---|---|---|---|---|---|---|
| 15·2 | 443·2(140) | 442·1(128) | + 1·1 | 1·0 | 33 | 33 |
| 18·2 | 253·5(215) | 249·9(196) | + 3·6 | 2·2 | 9 | 11 |
| 22·1 | 148·0(197) | 144·3(179) | + 3·7 | 8·4 | 9 | 12 |
| 25·4 | 110·8(186) | 105·7(170) | + 5·1 | 7·4 | 13 | 14 |
| 29·1 | 90·1(175) | 85·9(159) | + 4·2 | 11·1 | 10 | 10 |
| 30·8 | 84·8(192) | 79·9(175) | + 4·9 | 6·2 | 13 | 19 |
| 32·3 | 83·9(178) | 80·0(162) | + 3·9 | 6·8 | 16 | 14 |
| 34·0 | 85·6(198) | 86·3(175) | − 0·7 | 15·6 | 19 | 23 |
| 34·7 | 114·0( 18) | 116·7( 35) | − 2·7 | — | 70 | 70 |

Figures in brackets are the number of individuals contributing to each mean.

They are the "expected" frequencies as explained in footnote [4].

Significant differences in the time to complete development cannot be given directly but must be considered in the logarithmic scale.

When $P = 0{\cdot}01$ significant differences are given by:

$$d = 2{\cdot}576 \sqrt{\frac{0{\cdot}0009756 \times (n_1 + n_2)}{n_1 \times n_2}}$$

where d = significant difference on the logarithmic scale.

$n_1$ and $n_2$ = number of individuals contributing to the values of the two means being compared.

A difference of 6 is significant in any part of the mortality table at $P = 0{\cdot}01$.

* For the purpose of this experiment mortality is defined as the percentage of eggs which failed to hatch.

*Mean Differences in Duration of Developmental Period.*

In order to equalize the variance, the analysis of variance[4] for the hours required for development of the eggs at the two moisture levels and seven temperatures (15·2° C. to 32·3° C.), was made on the transformed logarithmic scale (cf. Cochran, 1938). The means shown in Table 3 are geometric means; they do not differ from the arithmetic means in any case by more than 0·7 p.c. In determining significant differences these mean values must first be converted to logarithms. The eggs developed in a significantly shorter time at the higher moisture level $M_2$ at every temperature up to 32·3° C.

*Interaction.*

Interaction between temperature and moisture was measured on the proportional basis (i.e. log scale), it being more reasonable in this case to measure proportional rather than absolute differences. Interaction was found to be significant. This means that the moisture treatment had not had the same proportionate effect at each temperature. However, an analysis of the components of interaction shows that the interaction factor is small.[5] There is no significant interaction ($P = 0{\cdot}01$) between the means of any two adjacent temperatures. The average interval between adjacent temperatures is three degrees (Table 4, column 1). Interaction is significant only when the temperature interval exceeds nine degrees (Table 4, column 3), and then not over the whole temperature range.

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[4] In the analysis of variance of non-orthogonal data such as the original data from which Table 2 was derived, the usual method of analysis is not applicable. As an exploratory test, Yates's approximate method of analysis (cf. Cornish, 1936) was used and indicated a significant interaction. In order to provide a more rigorous test of the interaction, recourse was had to the analysis of variance by "expected" class frequencies. For this purpose the class frequencies at the two moisture levels at each temperature were made proportional, as in tests of independence in contingency tables. These are the frequencies shown in Table 3. A $\chi^2$ test showed that the observed frequencies did not depart by more than errors of random sampling from the proportional frequencies calculated. It was possible, therefore, to proceed with the analysis of variance, in the usual manner, using the "expected" frequencies and "expected" class totals.

[5] It should be noted that significant differences between means and for interaction are both small. The intra class variance of $\log x = 0{\cdot}0009756$ (Table 3).

TABLE 4.

*Showing the distribution of components of interaction between temperature and moisture.*

| Average temperature intervals °C. | | | | |
|---|---|---|---|---|
| 3° | 6° | 9° | 12·5° | 15° |
| 15·2–18·2 | 15·2–22·1 | 15·2–25·4 | 15·2–29·1 | *15·2–30·8 |
| 18·2–22·1 | 18·2–25·4 | *18·2–29·2 | *18·2–30·8 | *18·2–32·3 |
| 22·1–25·4 | 22·1–29·1 | *22·1–30·8 | 22·2–32·3 | |
| 25·4–29·1 | 25·4–30·8 | 25·4–32·3 | | |
| 29·1–30·8 | 29·1–32·3 | | | |
| 30·8–32·3 | | | | |

* Interaction significant.

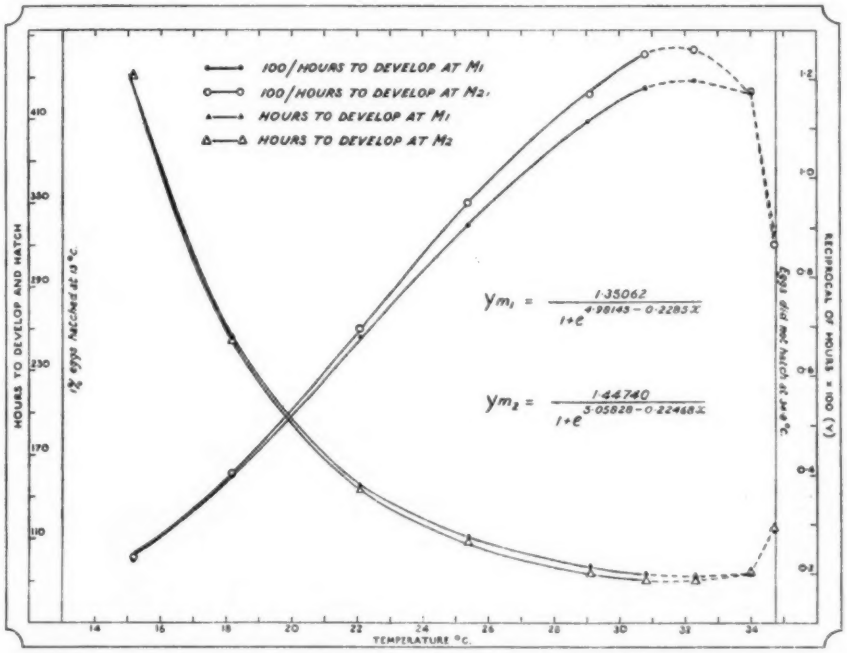
100/HOURS TO DEVELOP AT M1
100/HOURS TO DEVELOP AT M2
HOURS TO DEVELOP AT M1
HOURS TO DEVELOP AT M2
HOURS TO DEVELOP AND HATCH
% eggs hatched at t °C.
% eggs did not hatch at t °C.
RECIPROCAL OF HOURS = 100 (Y)
TEMPERATURE °C.
$y_{m_1} = \frac{1{\cdot}35062}{1+e^{4{\cdot}98145 - 0{\cdot}2285X}}$
$y_{m_2} = \frac{1{\cdot}44740}{1+e^{5{\cdot}05826 - 0{\cdot}22468X}}$

**Fig. 3.** Calculated curves showing the trend with temperature of time to develop and the reciprocal values of time to develop. Logistic curves were fitted to the values lying between 15·2° C. and 30·8° C.; the remaining part of the curve was drawn freehand. $M_1$ = moisture level corresponding to the value of saturation deficit × time = 0·96, $M_2$ = 0·56.

This analysis shows that the interaction between temperature and moisture is small when moisture is defined as the total evaporating power of the air (i.e. as the product of time × saturation deficit). This criterion for maintaining similar moisture conditions at different temperatures may be considered adequate. Complete absence of interaction is necessary for a perfect criterion.

*Mean differences in mortality.* There was no significant difference in mortality between moisture treatments at any one temperature, nor between the limits 18·2° and 32·3° C. Differences increase significantly outside these limits.

*Sigmoid Curve of the Reciprocal of the Time taken to Develop Plotted against Temperature.*

The reciprocal values of the time taken for the eggs to develop at the two levels of moisture have been plotted against temperature to give what is generally known as the "temperature-velocity" curves (Fig. 3). A logistic curve has been fitted to each set of points lying between the

temperatures 15·2° C. and 30·8° C. (cf. Davidson, 1942). The values of the constants in the curve $Y = \frac{K}{1 + e^{A + BX}}$ were first estimated approximately by graphical method (Davidson, 1944). First approximations to the maximal likelihood solutions for the constants were then calculated (cf. Appendix). The curves so derived have the form:

$$\text{Moisture level 1} \quad Y = \frac{1{\cdot}35062}{1 + e^{4{\cdot}98145 - 0{\cdot}22485X}}$$

$$\text{Moisture level 2} \quad Y = \frac{1{\cdot}44740}{1 + e^{5{\cdot}05828 - 0{\cdot}22468X}}$$

The observed and calculated values of the two curves are shown in Table 6. The difference between the observed and the calculated values are small in relation to the standard deviations of the observed values. It is not possible to give an overall "within arrays" variance because of the significant inequality of variance within temperature arrays. I am indebted to Mr. E. A. Cornish for the following approximate test of goodness of fit of the logistic curve to the observed data:

$$\text{Calculate } \chi^2 = \Sigma \frac{(\bar{y} - Y)^2}{\sigma^2}$$

where $\bar{y}$ = mean observed value,

$Y$ = calculated value,

$\sigma^2$ = variance within temperature array, and the summation is taken over all temperature arrays.

$\sigma^2$ has only two degrees of freedom and its values for the two curves are 0·1821 and 0·1901. These values lie between $P = 0{\cdot}95$ and $P = 0{\cdot}90$ so the trend of the curves is faithfully described by the logistic curves having the formulae given. The agreement between observed and calculated values is more readily appreciated when the differences are expressed as hours; at all temperatures the maximum difference is two hours except at the lowest temperature 15·2° C. where the difference was 12 hours.

It should be pointed out that the statistical analysis is approximate only. Sufficient detail has been given to enable the reader to carry out similar analyses to those made in this paper.

TABLE 6.

*Showing the goodness of fit of the calculated logistic curves.*

| Temperature. °C. | Moisture level 1. Observed. | Calculated. | Observed-calculated. | Standard deviation. | Moisture level 2. Observed. | Calculated. | Observed-calculated. | Standard deviation. |
|---|---|---|---|---|---|---|---|---|
| 15·2 | 0·226 | 0·233 | − 0·007 | 0·0175 | 0·227 | 0·234 | − 0·007 | 0·0172 |
| 18·2 | 0·395 | 0·394 | + 0·001 | 0·0245 | 0·401 | 0·398 | + 0·003 | 0·0208 |
| 22·1 | 0·677 | 0·671 | + 0·006 | 0·0372 | 0·694 | 0·690 | + 0·004 | 0·0360 |
| 25·4 | 0·905 | 0·911 | − 0·006 | 0·0660 | 0·948 | 0·950 | − 0·002 | 0·0592 |
| 29·1 | 1·114 | 1·114 | − 0·000 | 0·0881 | 1·169 | 1·176 | − 0·007 | 0·1014 |
| 30·8 | 1·184 | 1·181 | + 0·003 | 0·1080 | 1·251 | 1·253 | − 0·002 | 0·0936 |
| | | | − 0·002 | | | | − 0·011 | |

SUMMARY.

An experimental procedure has been described which gives precise information on the influence of temperature on the time taken for the eggs of the small strain of *Calandra oryzae* L. to develop.

The method of the experiments is based on the hypothesis that the influence of moisture on the development of the eggs of this species at different temperatures is the same when the eggs lose the same amount of water at these temperatures. That is, when the product of saturation deficit x duration of development x factor for air movement is constant. The small interaction between temperature and moisture indicated that the procedure was sound.

An apparatus for ensuring the circulation of air in a closed jar has been described.

The data obtained were analysed on the transformed logarithmic scale, using the approximate analysis of variance of Yates.

An increase in moisture corresponding to an increase of 10 p.c. R.H. at 75 per cent. and 25·4° C. gave a significantly faster rate of development at temperatures between 15° C. and 32·3° C. Interaction between temperature and moisture was significant when the temperature interval exceeded 9° C. Development took place in the shortest time (peak temperature) at 32·3° C.

Logistic curves have been fitted to the curves of reciprocal of time to complete development plotted against temperature. A means of testing the goodness of fit of these curves is given.

## APPENDIX.

Outline of procedure used in calculating the first approximations to the maximal likelihood solutions for the constants in the formula $Y = \frac{K}{1 + e^{A + BX}}$.

The normal equations to be solved are:

$$k\,S(x_1^2) + aS(x_1x_2) + bS(x_1x_3) = S(x_1z)$$
$$k\,S(x_1x_2) + aS(x_2^2) + bS(x_2x_3) = S(x_2z)$$
$$k\,S(x_1x_3) + aS(x_2x_3) + bS(x_3^2) = S(x_3z)$$

The above equations have to be solved for k, a and b, where k, a and b are the corrections to the provisional values $K_0$, $A_0$ and $B_0$ obtained by graphical method.

$x_1 = \frac{1}{1 + C}$, $x_2 = \frac{-K_0 C}{(1 + C)^2}$, $x_3 = \frac{-K_0 \times C}{(1 + C)^2}$, where $C = e^{A_0 + B_0 X}$ and X = temperature.

The summation of $x_1$, $x_2$ and $x_3$ is taken over all temperature arrays. $z = y - Y_0$ where y = value of the group intervals within each temperature array. $x_1z$, $x_2z$ and $x_3z$ are summations within each temperature array. $S(x_1^2)$, $S(x_1z)$ and $S(x_2z)$, etc., are summations taken over all temperature arrays.

*Acknowledgments.* Grateful acknowledgment is made to Professor J. Davidson and Mr. H. G. Andrewartha for help and guidance given in all aspects of the work. In the statistical treatment and curve fitting I have had the invaluable assistance of Mr. E. A. Cornish, Section of Mathematical Statistics of the Council for Scientific and Industrial Research.

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# DETOXICATION OF DIPHTHERIA TOXIN

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*(Accepted for publication 22nd September, 1944.)*

In previous communications (Petherick and Singer, 1943, 1944) a mechanism responsible for the detoxication of bacterial toxins by the simultaneous action of certain metals and redox substances or hydrogen peroxide has been described. This detoxication could be observed both *in vivo* and *in vitro*. The hypothesis has been advanced that this detoxication mechanism acts primarily on the amino groups of the toxin molecule, which are generally considered to be essential for the specific effect of bacterial toxins.

Detailed investigations of the changes occurring in the toxin molecule when exposed to metal ions and redox substances were, therefore, indicated. As large amounts of toxin are required for such investigations, the studies were restricted to the fate of diphtheria toxin when subjected to either an iron-ascorbic acid or copper-ascorbic acid system.

Numerous experimental investigations on the influence of ascorbic acid on crude toxic filtrates have appeared in the literature but the results of these investigations have been inconclusive. The literature on this subject is summarized by Willison (1943).

## MATERIAL AND METHODS.

Crude diphtheria toxin and standard flocculating immune serum were obtained through the courtesy of Dr. Morgan, Director of the Commonwealth Serum Laboratories, Melbourne. The crude toxin was partly purified according to the method of Eaton (1936). Solutions of toxin freed from bacillary protein and from a large part of non-specific broth constituents were readily obtained by this method. These solutions, which contained approximately 140 Lf per ml., were preserved with 1/10,000 merthiolate and kept in the ice-chest. Immediately before use appropriate dilutions with saline were made. All solutions were kept at pH 7·5.

Formol toxoid, also supplied by the C.S.L., was purified by the same process.

The metal salts used were B.D.H. Analytical Reagents. Ascorbic acid produced by the Commonwealth Sugar Refinery was used.

Flocculation tests were performed in a constant temperature bath with glass sides. The temperature was 50° C. and the tube racks were left undisturbed throughout the test to avoid convection currents which shorten the time of flocculation.

The flocculation tests were performed in test tubes of 1 cm. diameter, the volume of solution being 1 ml. The flocculating serum was added with a micro-burette. Differences of 2 Lf units could be observed readily and differences of 1 Lf unit could be judged with fair accuracy. The crude toxic filtrate contained 14 Lf units per ml. and the time of flocculation was 60 minutes. The time of flocculation was not increased by the purification process. If not otherwise stated the flocculation time remained constant in the experiments given in the Tables.

## EXPERIMENTAL.

As reported in a previous communication (Petherick and Singer, 1943) dilutions of diphtheria toxin containing 300 skin test units per ml. are partly detoxified when exposed to iron, copper or zinc and ascorbic acid, adrenaline or glutathione, the concentration of both metal ion and redox substance being 1 mg. p.c.

Quantitative investigations on the percentage destruction of the toxin had not been performed and it was necessary, therefore, to determine the minimum amount of ascorbic acid which would destroy a certain quantity of diphtheria toxin under standard conditions. A solution of diphtheria toxin containing 20 Lf units per ml. was selected for this experiment. Diphtheria toxin was mixed with a copper sulphate solution and solutions of neutralized ascorbic acid of varying

[1] Working under a full-time grant from the National Health and Medical Research Council.

concentrations. The final solution contained 20 Lf units of toxin per ml., 0·38 or 0·75 mg. p.c $Cu^{++}$ and from 700 mg. p.c. to 3,500 mg. p.c. ascorbic acid. Before the ascorbic acid was added it was neutralized with sodium hydroxide.

In order to compare these experiments with those reported previously it must be realized that in the latter, highly diluted solutions containing approximately 300 skin test units per ml. were used, whereas in the present experiments the concentration of toxin was 20 Lf units per ml., i.e. approximately 350,000 skin test units per ml. This was roughly a thousand-fold increase. The ascorbic acid concentration had, therefore, to be raised in proportion.

The results of an experiment designed to determine the amount of ascorbic acid necessary to destroy toxin under these conditions are given in Table 1.

TABLE 1.

*Influence on diphtheria toxin of incubation with copper salts and ascorbic acid for 4½ hours at 37° C.*

| Concentration of copper. mg. p.c. | Concentration of ascorbic acid. mg. p.c. | Lf value. Before incubation. | Lf value. After incubation. |
|---|---|---|---|
| — | — | 20 | 20 |
| 0·75 | 700 | . | 18 |
| 0·75 | 1,400 | . | <6 |
| 0·75 | 2,800 | . | <6 |
| 0·75 | 3,500 | . | — |

20 ml. of a solution which contained 20 Lf. units of diphtheria toxin per ml., 0·75 mg. p.c. $Cu^{++}$ and neutralized ascorbic acid to the concentration required were placed in a 100 ml. Erlenmeyer flask and incubated at 37° C. for 4½ hours. A toxin solution of the same concentration but without addition of copper or ascorbic acid was set up as control. The toxin content of the solutions was determined before and after incubation by the flocculation test.

When immune serum was added to toxin-copper-ascorbic acid mixtures in which the copper concentration was greater than 0·37 mg. p.c. the copper salt caused unspecific precipitation of the serum proteins.

On incubation however, the copper appeared to enter some combination in which it no longer caused this precipitation. Controls with normal horse serum and tetanus immune serum were performed to avoid possible errors due to non-specific precipitation. From the Table it can be seen that 700 mg. p.c. of ascorbic acid had only a slight effect on the toxin; the toxin concentration decreased from 20 Lf to 18 Lf per ml. When the ascorbic acid concentration was increased to 1,400 or to 2,800 mg. p.c. the toxin concentration fell below 6 Lf per ml. This concentration was the lowest which could be determined with accuracy. A concentration of ascorbic acid of 3,500 mg. p.c. led to complete destruction of the toxin. All tubes remained perfectly clear after addition of the anti-serum.

The possibility had to be considered that exposure to mixtures of ascorbic acid and copper would convert the toxin either into a substance of hapten character or alter its properties in so far that the toxin could still combine with antibody, but would be non-precipitable. Further, it had to be kept in mind that the medium might exert an unspecific inhibitory effect. To exclude these possibilities, the following experiments were performed.

20 ml. of a solution containing 20 Lf units of toxin per ml., 0·75 m.g. p.c. $Cu^{++}$ and 3,500 mg. p.c. neutralized ascorbic acid were incubated at 37° C. for 4½ hours. Undiluted purified toxin was then added to the mixture so that the final product would contain 18 Lf units per ml. of fresh toxin.

The results of these experiments excluded the possibilities considered above, as the freshly added toxin flocculated at the expected Lf value and in the expected time. The presence of a substance of hapten character or of a non-specific nature which could interfere with precipitation would have been disclosed by inhibition of flocculation. Presence of denatured toxin would have been disclosed by the "blending process", the number of Lf units increasing above the expected value. These results, therefore, furnish final proof that the diphtheria toxin exposed to the action of ascorbic acid and $Cu^{++}$ ions had been completely destroyed.

The experiments described above with copper and ascorbic acid were repeated substituting ferrous sulphate for copper sulphate. It was observed that in the majority of instances no decrease in the Lf value occurred. In a few experiments only a slight decrease was noted. The first impression gained was, therefore, that an iron-ascorbic acid system has no effect on diphtheria toxin. Previous experience, however, had shown clearly that combinations of $Fe^{++}$ and ascorbic acid have a detoxifying effect of the same order of magnitude as combinations of $Cu^{++}$ and ascorbic acid.

Moreover, in curative experiments in animals, injection of iron is much more effective than injections of comparable amounts of copper (Petherick and Singer, 1944). It was suspected,

therefore, that incubation with Fe ++ and ascorbic acid under the conditions of the experiment led to a change in the toxin not disclosed by the flocculation test but demonstrable in animal experiments. That such a change actually occurred was shown by results which are set out in Table 2.

TABLE 2.

*Detoxication of diphtheria toxin by iron or copper and ascorbic acid when incubated for 4½ hours at 37°C. Metal 0·75 mg. p.c., ascorbic acid 3,500 mg. p.c.*

| | Before incubation. | | After incubation. | |
|---|---|---|---|---|
| | Skin test unit ml. | Lf. | Skin test unit ml. | Lf. |
| Toxin | 0·000003 | 18 | 0·000003 | 18 |
| Toxin + Cu++ + ascorbic acid | 0·000003 | | >0·002 | — |
| Toxin + Fe++ + ascorbic acid | 0·000003 | 18 | 0·00001 | 18 |

Incubation with copper and ascorbic acid destroyed toxin completely; both combining power as measured by flocculation test and toxicity as measured by intradermal tests in guinea-pigs completely disappeared.

Incubation of toxin with the same concentration of ascorbic acid but substituting iron for copper led to a product which flocculated in some experiments at a slightly lower Lf value which at the most represented a 12 p.c. decrease in combining power. The toxicity, however, decreased to about 1/3rd the original value, the skin test dose increasing from the original 0·1 ml. of a 1/35,000 dilution to 0·1 ml. of a 1/10,000 dilution, which corresponds to a 70 p.c. decrease.

Using standard terminology, incubation of toxin with iron and ascorbic acid led to the production of a toxoid which retained only 1/3rd of the toxicity of the toxin. This result is compatible with the hypothesis that the detoxication begins at the amino groups of the toxin molecule.

An oxidative deamination or similar process leading to a product of toxoid character could be followed by determining the concentration of free ammonia in the toxin solutions before and after incubation with iron and ascorbic acid.

As already mentioned in a previous communication (Petherick and Singer, 1943), diphtheria toxin is so labile that even when buffered to a pH of 9·2 according to the method of Parnas, large amounts of ammonia are liberated. Hence a direct test by determination of free ammonia was not feasible.

Another test of the hypothesis could be performed by investigating the action of a Cu++-ascorbic acid mixture on formol toxoid. It is generally assumed that the amino groups essential for the toxic effect of diphtheria toxin react with formaldehyde with substitution of one or both hydrogen atoms by the $CH_2$ radical, and that this change is responsible for the loss of toxicity and preservation of combining power. Such conversion of the amino groups of the protein would render the substituted groups less liable to oxidation.

Some indication that the toxoid obtained by treating toxin with formaldehyde is more resistant is given by the fact that formol toxoid is less liable to deteriorate on storage, warming or during purification procedures than the corresponding toxin.

The result of an experiment demonstrating the resistance of formol toxoid when subjected to treatment with copper and ascorbic acid is given in Table 3. Final concentrations were, toxoid 22 Lf per ml., metal 0·75 mg. p.c., ascorbic acid, 3,500 mg. p.c.

TABLE 3.

*Effect on formol toxoid of incubation with iron or copper and ascorbic acid for 4½ hours at 37°C.*

| | Before incubation. | | After incubation. | |
|---|---|---|---|---|
| | Lf. | Kf. min. | Lf. | Kf. min. |
| Toxoid | 22 | 70 | 20 | 65 |
| Toxoid + Cu++ + ascorbic acid | 22 | 70 | 22 | 135 |
| Toxoid + Fe++ + ascorbic acid | 24 | 60 | 24 | 60 |

An amount of copper and ascorbic acid which was found regularly to be capable of destroying diphtheria toxin in a solution containing 20 Lf units per ml., had no influence on the combining power of an equivalent amount of toxoid under the same conditions. The results indicated, however, that the "toxophoric groups", assumed to be identical with the amino groups of the molecule, are not the only point of attack. Pronounced prolongation of the flocculating time of the toxoid after treatment with copper and ascorbic acid was observed. This indicated that, although the "haptophoric groups" of the toxoid had escaped destruction, partial denaturation of the toxoid molecule had occurred.

In experiments reported both here and previously, no destruction of toxin occurred when the toxin was incubated for a period of 4 to 5 hours with either iron or copper without addition of

hydrogen peroxide or a redox substance. Only those processes which are active within a few hours after injection of toxin can be considered to be of biological importance. At least part of diphtheria toxin injected either intradermally or intravenously remains free for this time. After this interval no free toxin can be demonstrated in extracts from the injected site after intradermal injection, or in the blood and organ extracts of animals which have been injected intravenously (Petherick, 1942).

Notwithstanding, it was of interest to investigate the question whether iron or copper, which were the two metals which showed highest activity in the detoxication of diphtheria toxin, could catalyse the detoxication of the toxin by molecular oxygen. Experiments were set up with the technique employed in previous investigations (Petherick and Singer, 1943), the only change in technique being the prolonging of the incubation period to 16 hours.

TABLE 4.

*Effect on diphtheria toxin of incubation with iron and copper, aerobically, for 16 hours.*

| Concentration of toxin (skin test units). | Salt tested. | Metal. mg. p.c. | No. of units injected intradermally. | Skin reaction. Before. incubation. | Skin reaction. After incubation. |
|---|---|---|---|---|---|
| 30 | — | — | 1 | + | ± |
| | | | 2 | ++ | + |
| 30 | $CuSO_4$ | 1·5 | 1 | + | — |
| | | | 5 | ++ | — |
| | | | 10 | ++ | ± |
| | | | 15 | ++ | ++ |
| 30 | $FeSO_4$ | 1·5 | 1 | + | ± |
| | | | 5 | ++ | + |
| | | | 10 | ++ | ++ |
| | | | 15 | ++ | ++ |

+ Signifies the reaction obtained with one skin test dose of diphtheria toxin.
± Signifies a reaction less than that with 1 skin test dose.
++ Signifies a reaction greater than that with 1 skin test dose.

A slight decrease only in toxicity occurred in toxin solutions incubated for 16 hours without the addition of metal salts, but there was a strong decrease in the toxin solution to which copper sulphate had been added. The decrease in toxicity of the solution to which ferrous sulphate had been added was not significantly greater than that of the corresponding toxin control without $FeSO_4$.

It appears, therefore, that the addition of small amounts of copper sulphate to the toxin solution catalyses the detoxication of toxin which occurs on exposure to air, and that under similar conditions iron in the form of $FeSO_4$ is ineffective. It must be realized, however, that ferous sulphate in neutral solutions exposed to air is quickly converted to insoluble basic ferric sulphate. This oxidation and consequent precipitation of the iron is probably the cause of the ineffectiveness of the added ferrous sulphate.

## DISCUSSION.

With serological methods it is possible to determine three different properties of diphtheria toxin which can, to a certain extent, change independently of each other.

The toxicity of the toxin as measured by the M.R.D. In our experiments an amount slightly higher than the M.R.D. has been used, namely, that amount of toxin which on intradermal injection in the guinea pig produces infiltration and redness of 1 cm. diameter after 48 hours; this is called the skin test dose.

The combining power as measured by the Lf value. These two properties can be regarded as specific properties of diphtheria toxin.

Unspecific denaturation of the toxin-protein caused by a variety of agencies is demonstrated in the flocculation test by a prolongation of the time of first particulation.

The experiments reported in this paper show that the effect of exposure of toxin solutions of uniform strength to standard amounts of neutralized ascorbic acid is dependent on the metal catalyst present in the reaction mixture.

$Fe^{++}$-ascorbic acid mixtures lead to marked detoxication with only slight loss of combining power.

$Cu^{++}$-ascorbic acid mixtures of the same concentration destroy the toxin completely.

The question as to whether this different effect is caused by a higher intensity of oxidation by the $Cu^{++}$-ascorbic acid mixtures or by a different point of attack constitutes a problem which would have to be investigated separately. From the fact that the combining power of formol toxoid exposed to ascorbic acid-$Cu^{++}$ mixtures, capable of destroying equivalent amounts of toxin, remains unaltered, it would appear that the main point of attack of both oxidation systems lies in the amino groups of the toxin molecule. Apart from this specific effect, nonspecific denaturation can occur as shown by prolongation of the flocculating time.

The same possibility is suggested by the results of experiments with $Fe^{++}$-ascorbic acid mixtures in which toxin could be detoxified without loss of combining power.

That addition of ascorbic acid to crude toxin broth results, in some instances, in the destruction of the toxin has long been known, and it has been forgotten, apparently, that this effect is not restricted to ascorbic acid; the detoxication of toxins by redox substances was first described by Marie (1918). He observed that addition of adrenalin to tetanus toxin resulted in loss of toxicity and that animals could be protected against a fatal dose of toxin by a previous injection of adrenalin.

Recently Willison (1943) discussed the possible importance of $Cu^{++}$ ions in the detoxication of crude toxic filtrates by ascorbic acid. Our results furnish proof that metal catalysts are essential for this process and that the effect of addition of ascorbic acid is dependent on the metal catalyst present.

The reverse, that is production of redox substances or hydrogen peroxide by the living diphtheria bacilli, is, in all probability, the explanation of the results of Pappenheimer and Johnson (1936), who found that the addition of iron to semi-synthetic media prevented production of toxin. Actually the addition of iron would not prevent toxin formation but would result in its continuous destruction by the simultaneous action of the metal catalyst and either redox substances or hydrogen peroxide. An additional effect might be due to catalysis of oxidation by oxygen from the air.

Our previous experiments have furnished evidence that the same or a similar process is responsible for detoxication of bacterial toxins *in vivo*.

## SUMMARY.

The detoxication of purified diphtheria toxin by iron or copper and ascorbic acid has been investigated.

Iron and neutralized ascorbic acid together produced detoxication but no change in combining power.

Incubation with copper and neutralized ascorbic acid led to complete destruction of toxin.

Formol toxoid was unaffected by a copper-ascorbic acid mixture sufficient to destroy equivalent amounts of unaltered toxin.

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# THE KINETICS OF FERMENTATION OF d-FRUCTOFURANOSE BY YEAST

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*(Accepted for publication 17th October, 1944.)*

Alcoholic fermentation of sugar by yeast cells consists of a series of successive enzyme reactions in which each reactant is the product of the preceding step. Moreover, some of the steps are interrelated by carrier systems. Thus, e.g., phosphorylation of the individual fermentable sugar at carbon atom six by adenosine triphosphate, a reaction catalyzed by hexokinase, is dependent on the phosphate transfer from 2-phosphopyruvate to adenosine diphosphate. If the concentration of the fermentable substrate within the cell is so high that all the enzyme molecules involved in the catalysis of the limiting reaction are tied up in the enzyme-substrate complex, the rate of fermentation is at its maximum; further addition of substrate has no effect on the velocity of the reaction. It is generally stated that d-glucose is fermented at about the same rate between 1 and 10 per cent. and d-fructose between 2 and 8 per cent. concentration the rate for fructose being only slightly less than that for glucose. (Slator, 1908; Hopkins and Roberts, 1935). The relative rates of consumption of d-glucose and of d-fructose from a mixture of both have been, however, found to be unequal. Brewery yeast (top and bottom), baker's yeast and most other yeasts prefer glucose to fructose, when offered in a mixture, a phenomenon known as "selective fermentation", first described by Dubrunfaut (1847) and confirmed by subsequent workers. (For literature *cf.* Sobotka and Reiner, 1930a). Willstätter and Sobotka (1922b) expressed the results of their experiments on selective fermentation by means of the formula $\frac{k_g}{k_f} = \frac{\ln a_o - \ln a}{\ln b_o - \ln b}$, where $a_o$ and $b_o$ are the initial concentrations of glucose and of fructose, a and b the concentrations of the respective sugars at any given time and where $\frac{k_g}{k_f}$ is the ratio of the separate rate constants of glucose and of fructose consumption from a mixture of both by yeast; it is assumed that in the mixed fermentation the rate of fermentation of each individual sugar is proportional to its concentration at any time. The factor $\frac{k_g}{k_f}$ (selectivity constant) varies between 2·0 and 3·0 for most of the yeast strains tested (Willstätter and Sobotka, 1922b; Hopkins, 1928 and 1931; Ivekovich, 1930; Sobotka and Reiner, 1930a). From these results the inference was drawn that the yeast enzyme concerned, probably the enzyme catalyzing the phosphorylation of the hexose, has a higher affinity for d-glucose than for d-fructose (Dawson, 1932). Later Hopkins and Roberts (1935) showed that the alcoholic fermentation of d-glucose and of d-fructose by living yeast proceeds according to the equation of Michaelis and Menten (1913). The value (2·1) of the ratio of the affinities of the yeast enzyme concerned for glucose and for fructose, arrived at by experimental determination of the Michaelis constants, was found to be in close agreement with that (2·3) of the selectivity constant derived from experiments on selective fermentation of a mixture of the sugars by a similar yeast. This seemed to be further and quantitative evidence for the assumption that the yeast enzyme has a higher affinity for d-glucose than for d-fructose.

All mathematical evaluations of experimental results with d-glucose, d-fructose or a mixture of both as the fermentable substrate are based on the assumption that d-glucose and d-fructose, when added to a yeast suspension, are each present exclusively in a fermentable form. This, however, holds only for d-glucose. For d-fructose it was recently shown (Gottschalk, 1943a) that only that fraction which exists in the furanose form undergoes fermentation, the pyranose modification, due to the lack of an OH-group at carbon atom six, being unfermentable. If, therefore, the rate of fermentation of d-fructose is determined and compared with that of d-glucose, it must be remembered that the actual concentration of fermentable fructose, i.e. of d-fructofuranose, is only a small portion of the total concentration of d-fructose. At 20° C. (pH = 4·3) a d-fructose solution at equilibrium contains approximately 20 p.c. of the sugar in the furanose form (Gottschalk, 1943b).

Hence it seemed appropriate to investigate the kinetics of fermentation of d-fructofuranose by yeast cells. In the experiments described in this paper the rates of fermentation over a wide range of concentrations of d-fructofuranose and of α-d-glucose were determined using different strains of *Saccharomyces cerevisiae* and *S. ellipsoideus*. The results, thus obtained, were evaluated by application of the theorem of Michaelis and Menten (1913).

## EXPERIMENTAL.

The experiments were performed with yeast prepared in the laboratory from pure cultures of *Saccharomyces cerevisiae* (power-alcohol strain) and of *S. ellipsoideus* (port-wine and champagne strains) as well as with brewery top and bottom yeast. From the power-alcohol and port-wine strains single-cell cultures were established. Cultures were grown at 25° C. in commercial malt extract which had been diluted with phosphate buffer (pH = 6·1) and sterilized by filtration through Seitz pads. After two 48 hour passages through 10 ml. and 100 ml. of medium respectively strains were sown into a series of 20 oz. "medicine flats" each containing 100 ml. of medium. To give increased aeration, the bottles were placed horizontally and shaken several times daily. After incubation for two days the united contents of these bottles were centrifuged at 5° C., washed twice on the centrifuge, once on the Büchner funnel and then compressed. The freshly collected brewer's yeast was thoroughly washed on a big Büchner funnel before compressing. The dry weight of the yeasts thus obtained varied between 26·5 and 27·9 p.c.

0·8–2·0 gm. of the yeast were suspended in M/20 $KH_2PO_4$; total volume = 100 ml.; pH of the suspension = 4·5 at the beginning of the experiment. 20·0 ml. of this suspension was used in each experiment.

The rate of fermentation was determined with the apparatus described previously (Gottschalk and Rawlinson, 1942). Fermentation was carried out at 25·0° C. ± 0·1 under strictly anaerobic conditions with nitrogen (free from oxygen) in the gas-space (volume of gas-space at the beginning of the experiment about 55 ml.). After equilibration with gas and after thermal equilibration the sugar placed in a small glass container was dipped into the yeast suspension and the increase in volume due to $CO_2$ production measured in intervals of five minutes for a period of 40–70 minutes, the values observed being reduced to S.T.P. The bulb containing the yeast suspension was shaken at a rate of 160 oscillations per minute.

For each type of yeast the rate of fermentation was determined with various initial substrate concentrations ranging from 0·0056 M to 0·2978 M d-glucose and from 0·0022 M to 0·0756 M d-fructofuranose. Four experiments with the same yeast suspension, freshly prepared each morning and stored at 10° C. until used, were performed per day, the next series being carried out the following day with overlapping sugar concentrations to eliminate rate differences due to ageing or loss of water of the stock yeast at 1–2° C.

The α-d-glucose and β-d-fructose (pyranose) used in the investigations were the same preparations referred to in a previous paper (Gottschalk, 1943a). The α-d-glucose was added in the solid state, unless otherwise stated. The d-fructopyranose was dissolved in 0·5–1·6 ml. M/20 $KH_2PO_4$ solution and allowed to mutarotate overnight at room temperature and early in the morning at 25° C. The fructofuranose content of the solution was calculated on the assumption that a d-fructose solution at equilibrium at 25° C. contains approximately 22 p.c. of the sugar in the furanose form. This figure was obtained from a consideration of the proportion of the furanose form in an equilibrium mixture of d-fructose at 0° C., as determined by experiment, and of the known differences in mutarotation of d-fructopyranose at 0° C. and at 25° C.

## CALCULATION OF THE RESULTS.

Hopkins and Roberts (1935) have first applied the theorem of Michaelis and Menten (1913) to the velocities of glucose fermentation by yeast observed with various initial concentrations of the substrate. By plotting $\frac{1}{v}$ against $\frac{1}{[S]}$ a straight line was obtained conforming to the theorem:

$$\frac{1}{v} = \frac{K_m}{V} \cdot \frac{1}{[S]} + \frac{1}{V}, \qquad [1]$$

where v = measured velocity of the reaction, i.e. rate of fermentation,

V = maximum velocity of the reaction which is reached, when the substrate concentration is high enough to tie up all the enzyme molecules in the enzyme-substrate complex (ES),

[S] = concentration of substrate,

$K_m$ = dissociation constant of the enzyme-substrate complex, also called the Michaelis constant; numerically it equals the substrate concentration at which $v = \frac{1}{2} V$. The reciprocal of $K_m$ is the affinity constant.

In the present paper the Michaelis and Menten equation was adopted in the form recommended by Lineweaver and Burk (1934), namely

$$\frac{[S]}{v} = \frac{[S]}{V} + \frac{K_m}{V}. \qquad [2]$$

From the experimental data for the rates of fermentation with various initial hexose concentrations the linear regression of $\frac{[S]}{v}$ on [S] was investigated; the regression coefficients, $\frac{1}{V}$ and $\frac{K_m}{V}$, thus obtained, were tested for significance in accordance with standard statistical technique.

From the figures set out in Table 2 and also in Table 2 of Hopkins' paper (1935, p. 932) it may be seen that with a given substrate concentration the rate of fermentation rises quickly in the initial period and then approaches asymptotically its highest value, as is to be expected from the equation given for the rate of fermentation by Weichherz (1929). In higher concentration of the substrate this value can be determined accurately, since then fermentation proceeds over a long period without much impairing the sugar concentration in the suspension fluid. With lower initial concentration, however, the decrease in substrate concentration due to sugar consumption causes the fermentation rate to fall off after a certain time depending on the initial substrate concentration and the amount of yeast. For this reason the highest rate of $CO_2$ production (ml. $CO_2$ per 5 min.) attained in the first 30 minutes of each experiment was taken as the fermentation rate for the given substrate concentration. Though after 30 minutes—with the yeast strains used in the experiments—the fermentation rate for a given substrate concentration is already very close to its highest attainable value, it may be that an unavoidable error is introduced by this procedure. It would appear, however, that this error is small, since the value for the ratio $V_f : V_g$ obtained by this procedure from the whole set of figures of an experiment agreed very well with that determined directly from the highest values reached in an independent pair of experiments with high concentrations of d-fructofuranose and of d-glucose respectively lasting 60–70 minutes; e.g. for power alcohol yeast 1·06 and 1·09, for wine yeast 1·45 and 1·41.

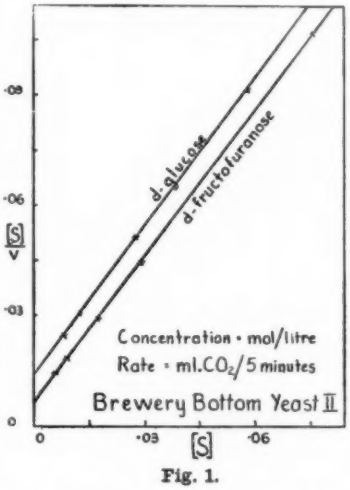

Fig. 1.

With d-fructofuranose as substrate an initial concentration as low as 0·0022 M could be employed, since in this case the decrease by fermentation in the concentration of the fermentable sugar is much slower than with a corresponding concentration of α-d-glucose; this is due to the

fact that part of the fructofuranose consumed is continuously restored by the equilibrium reaction fructopyranose $\rightleftharpoons$ fructofuranose; $(k_1 + k_2) \times 10^3 = 87$ at 25°C. and pH = 3·4 (Nelson and Beegle, 1919).

As shown in previous papers (Gottschalk, 1941 and 1942) only part of the sugar consumed by yeast is fermented to $CO_2$ and alcohol, another part being assimilated. The results recorded in Table 1 indicate that the percentage of hexose fermented by the different types of yeast rises only slightly with increasing substrate concentration; where necessary, a small correction was applied.

## RESULTS.

### TABLE 1.

*Determination of the ratio* $\frac{\textit{hexose fermented}}{\textit{hexose consumed}}$ *at various initial concentrations of d-glucose and of d-fructose by different types of yeast.*

| Type of yeast. | Hexose used. | Amount added. mg. | Hexose fermented.* mg. | Hexose fermented / Hexose consumed. p.c. |
|---|---|---|---|---|
| Brewery top yeast (0·40g) | d-glucose | 20·3 | 17·0 | 83·7 |
| | d-glucose | 121·9 | 106·1 | 87·0 |
| Brewery bottom yeast (0·40g) | d-glucose | 50·0 | 41·2 | 82·4 |
| | d-glucose | 151·3 | 129·4 | 85·5 |
| | d-glucose | 204·6 | 176·0 | 86·0 |
| Brewery bottom yeast (0·30g) | d-glucose | 22·3 | 18·4 | 82·5 |
| | d-glucose | 100·0 | 85·2 | 85·2 |
| | d-fructose | 24·3 | 20·3 | 83·5 |
| | d-fructose | 99·2 | 85·1 | 85·8 |
| Power alcohol yeast (0·40g) | d-glucose | 21·9 | 17·9 | 81·7 |
| | d-glucose | 121·6 | 106·0 | 87·2 |
| | d-fructose | 20·8 | 17·4 | 83·6 |
| Wine yeast (Port) (0·35g) | d-glucose | 20·4 | 15·8 | 77·5 |
| | d-glucose | 149·0 | 122·6 | 82·3 |
| | d-fructose | 25·7 | 19·7 | 76·7 |
| | d-fructose | 150·0 | 120·5 | 80·3 |

* Fermented to the complete removal of the hexose added.

### TABLE 2.

*Ml. of $CO_2$ produced in successive five minute periods during the fermentation of α-d-glucose and of d-fructofuranose at various initial concentrations.*

(Brewery bottom yeast II).

| Initial hexose concentration. moles/litre. | Periods in minutes. 0–5. | 5–10. | 10–15. | 15–20. | 20–25. | 25–30. | 30–35. | 35–40. |
|---|---|---|---|---|---|---|---|---|
| α-d-glucose | | | | | | | | |
| 0·0081 | 0·24 | 0·33 | 0·33 | 0·33 | 0·33 | 0·30 | 0·27 | 0·27 |
| 0·0124 | 0·24 | 0·36 | 0·36 | 0·41 | 0·41 | 0·41 | 0·41 | 0·37 |
| 0·0278 | 0·36 | 0·48 | 0·54 | 0·54 | 0·53 | 0·53 | 0·53 | 0·56 |
| 0·0567 | 0·49 | 0·60 | 0·60 | 0·62 | 0·62 | 0·62 | 0·60 | 0·65 |
| 0·2828 | 0·55 | 0·67 | 0·66 | 0·72 | 0·72 | 0·71 | 0·74 | 0·74 |
| d-fructofuranose | | | | | | | | |
| 0·0059 | 0·35 | 0·35 | 0·38 | 0·40 | 0·40 | 0·40 | 0·40 | 0·40 |
| 0·0087 | 0·41 | 0·42 | 0·47 | 0·47 | 0·47 | 0·47 | 0·46 | 0·46 |
| 0·0172 | 0·36 | 0·59 | 0·59 | 0·59 | 0·58 | 0·58 | 0·58 | 0·61 |
| 0·0287 | 0·48 | 0·59 | 0·65 | 0·65 | 0·64 | 0·64 | 0·68 | 0·69 |
| 0·0756 | 0·48 | 0·66 | 0·71 | 0·71 | 0·70 | 0·71 | 0·75 | 0·75 |

In Table 2 full details of a complete set of experiments with brewery bottom yeast II are given. In this case the equations of the regression line with d-fructofuranose and with α-d-glucose respectively as substrate are:

(fructofuranose) $Y = 0{\cdot}04262 + 1{\cdot}3165\ (x - 0{\cdot}02722)$
(glucose) $Y = 0{\cdot}1181 + 1{\cdot}3396\ (x - 0{\cdot}07756)$.

The estimates of the variances of a and b and the corresponding values for t, each for 3 degrees of freedom, are tabulated below:

| | Variance of a. | t. | Variance of b. | t. |
|---|---|---|---|---|
| d-fructofuranose | $2{\cdot}09022 \times 10^{-5}$ | 294·8 | $3{\cdot}225 \times 10^{-6}$ | 231·8 |
| α-d-glucose | $1{\cdot}6969 \times 10^{-7}$ | 286·7 | $1{\cdot}568 \times 10^{-6}$ | 338·3 |

In Fig. 1, plotting $\frac{[S]}{v}$ against [S], the experimental values are superimposed on the best fitting straight line given by the equation of the corresponding regression line.

TABLE 3.

*Rates of fermentation with various initial concentrations of α-d-glucose and of d-fructofuranose by different types of yeast.*

Concentration: moles per litre. Rate: ml. $CO_2$ per 5 minutes.

| Type of yeast. | Hexose used. | | | | | | |
|---|---|---|---|---|---|---|---|
| Brewery bottom yeast I | α-d-glucose | Conc.: | 0·0071 | 0·0086 | 0·0142 | 0·0287 | 0·0561 | 0·2861 |
| | | Rate: | 0·52 | 0·58 | 0·76 | 0·98 | 1·15 | 1·35 |
| | d-fructofuranose | Conc.: | 0·0022 | 0·0043 | 0·0088 | 0·0176 | 0·0338 | |
| | | Rate: | 0·40 | 0·65 | 0·91 | 1·15 | 1·30 | |
| Brewery bottom yeast II | α-d-glucose | Conc.: | 0·0081 | 0·0124 | 0·0278 | 0·0567 | 0·2828 | |
| | | Rate: | 0·33 | 0·41 | 0·54 | 0·62 | 0·72 | |
| | d-fructofuranose | Conc.: | 0·0059 | 0·0087 | 0·0172 | 0·0287 | 0·0756 | |
| | | Rate: | 0·40 | 0·47 | 0·59 | 0·65 | 0·71 | |
| Brewery top yeast | α-d-glucose | Conc.: | 0·0072 | 0·0099 | 0·0144 | 0·0290 | 0·0578 | 0·2978 |
| | | Rate: | 0·52 | 0·60 | 0·70 | 0·83 | 0·91 | 1·00 |
| | d-fructofuranose | Conc.: | 0·0042 | 0·0084 | 0·0169 | 0·0656 | | |
| | | Rate: | 0·58 | 0·76 | 0·91 | 1·03 | | |
| Power alcohol yeast | α-d-glucose | Conc.: | 0·0066 | 0·0137 | 0·0277 | 0·0561 | | |
| | | Rate: | 0·65 | 0·76 | 0·82 | 0·86 | | |
| | d-fructofuranose | Conc.: | 0·0022 | 0·0044 | 0·0087 | 0·0177 | 0·0325 | |
| | | Rate: | 0·59 | 0·74 | 0·82 | 0·88 | 0·91 | |
| Wine yeast (champagne) | α-d-glucose | Conc.: | 0·0056 | 0·0083 | 0·0140 | 0·0279 | 0·0558 | |
| | | Rate: | 0·47 | 0·53 | 0·60 | 0·68 | 0·73 | |
| | d-fructofuranose | Conc.: | 0·0022 | 0·0045 | 0·0090 | 0·0173 | 0·0349 | |
| | | Rate: | 0·62 | 0·80 | 0·94 | 1·02 | 1·07 | |
| Wine yeast (port) | α-d-glucose | Conc.: | 0·0057 | 0·0142 | 0·0278 | 0·0558 | | |
| | | Rate: | 0·52 | 0·68 | 0·74 | 0·78 | | |
| | d-fructofuranose | Conc.: | 0·0022 | 0·0046 | 0·0090 | 0·0174 | 0·0349 | |
| | | Rate: | 0·68 | 0·87 | 0·99 | 1·07 | 1·12 | |

In Table 3 the results of experiments with different types of yeast are shown; for each set of experiments the substrate concentrations and the corresponding rates of fermentation are recorded. In all these experiments the t-values obtained lie beyond the 0·001 level of significance. It follows that the regression between $\frac{[S]}{v}$ and [S] is linear, and accordingly we may conclude that the fermentation of d-fructofuranose and of d-glucose conforms to the theory of Michaelis and Menten.

TABLE 4.

*Values for the dissociation constants and the maximum velocities calculated from the experimental results shown in Table 3.*

| Type of yeast. | | $K_g$. | $K_f$. | $\frac{K_g}{K_f}$. | $V_f$. | $V_g$. | $\frac{V_f}{V_g}$. |
|---|---|---|---|---|---|---|---|
| Brewery bottom I | *S. cerevis.* | 0·0123 | 0·0061 | 2·0 | 1·54 | 1·41 | 1·09 |
| Brewery bottom II | *S. cerevis.* | 0·0105 | 0·0052 | 2·0 | 0·76 | 0·74 | 1·03 |
| Brewery top | *S. cerevis.* | 0·0069 | 0·0036 | 1·9 | 1·09 | 1·02 | 1·07 |
| Power alcohol | *S. cerevis.* | 0·0025 | 0·0013 | 1·9 | 0·95 | 0·90 | 1·06 |
| Wine (champagne) | *S. ellips.* | 0·0039 | 0·0018 | 2·2 | 1·13 | 0·78 | 1·45 |
| Wine (port) | *S. ellips.* | 0·0032 | 0·0016 | 2·0 | 1·17 | 0·83 | 1·41 |

In Table 4 the values for $K_g$, $K_f$, $V_g$ and $V_f$ and for the ratio of the Michaelis constants and of the maximum velocities are given.

In confirmation of the results of Hopkins and Roberts (1936) it was found that with low substrate concentrations the α-modification of d-glucose is fermented at a higher rate than the β-modification. With an initial concentration of 0·007 M the rate of fermentation of α-d-glucose is 10 p.c. higher than that of equilibrium-d-glucose (Table 5). If α-d-glucose is dissolved in M/20 $KH_2PO_4$ solution (pH = 4·5) at 25° C., about 30 p.c. of the sugar present is interconverted to the β-modification within 30 minutes; $(k_1 + k_2) \times 10^3 = 10{\cdot}4$ at 25° C. (pH = 4·3) (Nelson and Beegle, 1919).

TABLE 5.

*Ml. of $CO_2$ produced in successive five minute periods during the fermentation by yeast of α-d-glucose and of equilibrium-d-glucose.*

0·4 gm. brewery bottom yeast in M/20 $KH_2PO_4$ solution. Total volume: 20·0 ml.

α-d-glucose was added in the solid state to the yeast suspension; equilibrium-d-glucose was obtained by dissolving α-d-glucose in 0·5 ml. M/20 $KH_2PO_4$ and allowing it to equilibrate for 24 hours at 20° C.

| d-glucose used. | | Periods in minutes. | | | | |
|---|---|---|---|---|---|---|
| Modification. | Amount mg. | 0–5. | 5–10. | 10–15. | 15–20. | 20–25. |
| α-d-glucose | 25·4 | 0·47 | 0·52 | 0·52 | 0·52 | 0·46 |
| equilibrium-d-glucose | 24·5 | 0·48 | 0·47 | 0·47 | 0·47 | 0·35 |
| α-d-glucose | 51·2 | 0·70 | 0·76 | 0·76 | 0·74 | 0·69 |
| equilibrium-d-glucose | 50·9 | 0·59 | 0·71 | 0·70 | 0·70 | 0·64 |
| α-d-glucose | 103·2 | 0·70 | 0·98 | 0·98 | 0·97 | 0·95 |
| equilibrium-d-glucose | 102·2 | 0·71 | 0·95 | 0·94 | 0·88 | 0·92 |

## DISCUSSION.

The fact that the alcoholic fermentation of d-fructofuranose and of d-glucose, as determined by the rate of $CO_2$ production, conforms to the theorem of Michaelis and Menten suggests that a single enzyme reaction controls the rate of the whole process. Obviously, in a series of successive enzyme reactions, where all the reactants have a high affinity for the enzymes concerned, the over-all rate is limited by the step with the lowest value for V. Since the maximum velocities of the separate fermentations of d-fructofuranose and of d-glucose by the same sample of yeast differ, as is evident from the experiments with *S. ellipsoideus* (Table 4), none of the enzyme reactions common to both can limit the rate. It must, therefore, be concluded that one of those steps which in the fermentation of fructofuranose and of glucose precede the common chain of reactions controls the fermentation rate. There is only one reaction, the first one, which is different in fructofuranose and in glucose fermentation. All the other reactions are common steps including the equilibrium reaction glucose–6–phosphate⇌ fructofuranose–6–phosphate. Thus, Lohmann (1933) has demonstrated that this equilibrium is established within a few seconds when the aldose–6–ester or the ketose–6–ester is added to tissue ex-

tracts or dried yeast. The extremely high activity of the isomerase is also revealed by the fact that with equal amounts of d-glucose and of d-fructose added separately to yeast cells the same percentage of the hexose consumed is assimilated (Table 1) and the same percentage of the hexose consumed but not fermented is synthesized to glycogen and other polysaccharides (Gottschalk, 1941 and 1942). With d-fructose as substrate the reaction fructofuranose–6–phosphate → glucose–6–phosphate is an intermediate step in the formation of glycogen.

It follows that it is the initial step which determines the rate of alcoholic fermentation. The rate of $CO_2$ production is, therefore, a measure of the enzyme reaction

$$E + \text{hexose } (+ATP) \rightleftharpoons ES \xrightarrow{k} E + \text{hexose-6-phosphate } (+ADP) \quad [3]$$

where E = hexokinase, ES = hexokinase-substrate complex, k = velocity constant of the break-up of ES, ATP and ADP = adenosine triphosphate and diphosphate. By $K_g$ and $K_f$ (Table 4) are designated the dissociation constants of the hexokinase-substrate complexes when α-d-glucose and d-fructofuranose respectively are the substrates. $V_g$ and $V_f$ are the maximum velocities of the respective reactions. i.e. if $[E] = [ES]$; in this case $V = k \times [E]$ [4]

As may be seen from Table 4 the values for $K_g$ and $K_f$ differ considerably with different types of yeast, though only slightly with different samples of the same type. The ratio $K_g/K_f$, however, is constant within the limits of the experimental error for all types of yeast. *The average value of 2 for this ratio means that d-fructofuranose has twice the affinity of α-d-glucose for hexokinase.* For the strains of *S. cerevisiae* tested the ratio of the maximum velocities is constant and equals about 1·05. It is only in low concentrations of d-fructofuranose and of α-d-glucose that their different affinities for hexokinase become apparent (*cf.* equations [1] and [4]). The same holds for α- and β-glucose. In high concentrations (0·056 M and more) α- and β-glucose are fermented separately at the same rate (Willstätter and Sobotka, 1922a; Hopkins and Roberts, 1936). The greater affinity for hexokinase of α-d-glucose compared with that of β-d-glucose reveals itself only in low concentrations of the substrates, if fermented separately (*cf.* Table 5), or in the fermentation of a mixture of the two d-glucose modifications at any concentration (Willstätter and Sobotka, 1922a; Hopkins, 1931); in the latter case the affinity is one factor controlling the distribution of the enzyme between its two substrates.

In remarkable contrast to the constancy of the ratio $K_g/K_f$ for all types of yeast the ratio $V_f/V_g$ is much higher with *S. ellipsoideus* than with *S. cerevisiae*. The difference in the ratio is far beyond the limits of experimental error, is found in 30 min. and in 60 min. experiments and is not due to differences in the permeability of the two species of yeast for d-glucose and for d-fructofuranose, as was ascertained in two experiments in which the fermentation rates of d-fructofuranose and of d-glucose with fresh and with air-dried Champagne yeast were compared. If the above results are taken in conjunction with those obtained from experiments with Sauterne yeast (Sobotka and Reiner, 1930b; Hopkins, 1931), it seems to be a characteristic of many wine yeasts (*S. ellipsoideus*) that the maximum velocity of fermentation of fructofuranose ($V_f$) is 40–50 p.c. higher than that of d-glucose ($V_g$).

Since $V = k \times [E]$, the variation observed with different species of yeast in the ratio $V_f/V_g$ may be ascribed to variation either in k or in [E]. It seems improbable that any factor which affects the velocity constant of the break-up of the hexokinase-fructofuranose-phosphate complex would not affect or would affect differently the velocity constant of the break-up of the corresponding hexokinase-glucose-phosphate complex. Thus, Kuhn (1923) has established for saccharase preparations from different yeasts acting on sucrose and on raffinose respectively

that $K_r/K_s$ and $V_s/V_r$ are constant though the individual values for $K_s$ and $K_r$ vary considerably with different yeasts.

The alternative interpretation of the variation in the ratio of the maximum velocities is much more satisfactory. The suggestion is made that hexokinase is not a single enzyme, but an enzyme complex containing a specific "fructokinase" and a specific "glucokinase" for the transfer of a mobile phosphate group from ATP to d-fructofuranose and d-glucose respectively. It would then appear that the ratio $\frac{[\text{Fructokinase}]}{[\text{Glucokinase}]}$ is greater in *S. ellipsoidus* than in *S. cerevisiae*. The assumption of the existence of two specific kinases has been substantiated in recent experiments. In experiments with yeast juice prepared from dried yeast (maceration extract) it was found possible to differentiate between fructokinase and glucokinase. The details will be published shortly.

Though possessing specific kinases for the initial step in alcoholic fermentation, living yeast when acting on a mixture of d-glucose and d-fructofuranose does not ferment at a rate equal to the sum of the rates in separate fermentation. This is due to the fact that in such a mixed fermentation a common step or factor, possibly the amount of phosphate carrier available in the cell, limits the rate, thus preventing a full additive effect. To some extent, however, such an additive effect can be observed. In experiments with baker's yeast (200 mg. fresh yeast suspended in 19·8 ml. M/20 $KH_2PO_4$ solution) acting on 600 mg. α-d-glucose and 570 mg. sucrose respectively for 50 min. at 25° C. (in nitrogen) the ratio of the fermentation rates (ml. $CO_2$/5 min.) was found to be 0·83 : 0·90. Since $V_g$ in this case was 0·85 the increase above this level implies the activity of an additional enzyme. Slator (1906), Trautwein and Wassermann (1929), Sobotka and Reiner (1930a) have published very similar figures for the ratio of the fermentation rates by yeast with glucose and sucrose in high concentrations as substrates.

The "selective fermentation" of d-glucose from a mixture of d-glucose and d-fructose, described in the literature since 1847, is only apparent, its interpretation being based on the assumption that all fructose modifications present in a solution of d-fructose are fermentable. The ratio $\frac{v_g}{v_f}$ of the rates, at which d-glucose and d-fructofuranose may be expected to be fermented from a mixture of equal parts of d-glucose and d-fructose (at 25° C.), can be calculated by applying equation [5] and inserting for $K_f/K_g$ and $V_g/V_f$ the values obtained in this paper with *S. cerevisiae*:

$$\frac{v_g}{v_f} = \frac{[\text{Glucose}]}{[\text{Fructofuranose}]} \times \frac{K_f}{K_g} \times \frac{V_g}{V_f} = 2{\cdot}2 \qquad [5]$$

The value of 2·2 is in close agreement with the values of 2·07 to 2·30 given by Sobotka and Reiner (1930b) and Hopkins (1931) for the "selectivity constant", when a mixture of equal parts of d-glucose and d-fructose is fermented at 25° C. by brewery yeast to an extent of about 22 p.c. of the total sugar added. Thus, it is not the d-glucose, but the d-fructofuranose which is preferentially fermented from a mixture of both.

## SUMMARY.

The rates of fermentation by *S. cerevisiae* and *S. ellipsoideus* of d-fructofuranose and of α-d-glucose over a wide range of concentrations were determined. Both the fermentation of fructofuranose and of glucose conform to the theorem of Michaelis and Menten. Since experiments with *S. ellipsoideus* have shown that the first step in alcoholic fermentation controls the over-all rate, the rate of $CO_2$ production is a measure of the velocity of the enzyme reaction catalyzed by hexokinase. While the values for $K_g$ and $K_f$ (dissociation constants of the hexokinase-

glucose and of the hexokinase-fructofuranose complex) vary considerably with the different types of yeast, the ratio $K_g/K_f$ is constant for all yeast strains investigated. Its value is about 2·0, indicating that d-fructofuranose has twice the affinity of α-d-glucose for hexokinase. The ratio of the maximum velocities $V_f/V_g$ equals 1·05 for *S. cerevisiae* and approximately 1·45 for *S. ellipsoideus*. These values suggest that hexokinase is an enzyme complex containing in varying proportions a specific "fructokinase" and a specific "glucokinase" for the phosphate transfer from ATP to d-fructofuranose and d-glucose respectively. This assumption was substantiated by experiments with yeast extract.

*Acknowledgments.* My thanks are due to Dr. A. W. Turner, officer in charge of the Animal Health Laboratory, C.S.I.R., Melbourne, for his ready and valuable help in preparing the pure yeast cultures used in the experiments. I am also indebted to Dr. A. R. Callaghan, Principal of Roseworthy Agricultural College, South Australia, for his kindness in supplying the stock cultures of *S. ellipsoideus*.

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# LACK OF INCREASE IN ANTIBODY AFTER SECOND INJECTION OF INFLUENZA VIRUS IN MAN

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*(Accepted for publication, 2nd October, 1944.)*

It is well known that nearly all adults have at least a trace of antibody against influenza viruses A and B owing to past infection and that a single subcutaneous injection of a suitably potent vaccine consisting of formolized virus produces a significant increase in antibody in most persons. The response to one injection has been so satisfactory that there has been little investigation of the effect of a second injection. Stuart-Harris, Andrewes and Smith (1938) reported that in 15 men who were given two subcutaneous injections of influenza virus A the final antibody level was no higher than in 15 men who received only one inoculation. Hare, Morgan, Jackson and Stamatis (1943) injected four groups of people with formolized virus and a week later again inoculated two of the groups with the same vaccine and the other two groups with saline. Blood specimens taken one week after the final injection showed that the groups given two doses of vaccine had a somewhat lower mean titre than those receiving only one. However, no conclusions are justified from this observation because the final serum specimen was taken only one week after the second inoculation and it has been shown that after subcutaneous inoculation with influenza virus in man most of the increase does not usually occur until after the seventh day (Horsfall, Lennette and Rickard, 1941).

We were led to study the response to second injections of influenza virus by failure of an attempt to produce hyperimmune serum in man. Two individuals each given four injections showed no increase in antibody beyond the initial level. Both had received an inoculation several months previously.

## METHODS.

*Vaccine.* Two strains of virus were used, the BEL strain of influenza A in its 42nd embryo passage and the LEE strain of influenza B in its 40th embryo passage. Ten-day embryos were inoculated into the allantoic cavity with allantoic fluid virus diluted 1: 100. After 42 hours incubation at 35° C. the eggs were chilled for several hours and the allantoic fluid harvested. After tests for potency by haemagglutination and bacterial sterility tests, 0·025 p.c. formalin (0·01 p.c. HCHO) was added and the virus incubated 1 hour at 37° C. before storage in the refrigerator. The pooled virus used had a haemagglutination titre of 300–500 in the case of BEL and 120–200 in the case of LEE. Our general experience is that these virus preparations invariably have a titre of between $10^7$ and $10^8$ infective doses in 0·05 ml. inoculated into the allantoic cavity, but the actual batches used for the vaccine were not tested in this way. Equal volumes of BEL and LEE were mixed for all injections. No appreciable deposit formed on storage.

*Antibody titrations.* Specimens of blood for sera were taken at the time of inoculation and two weeks after each inoculation. They were heated at 56° C. for 30 minutes and titrated by the modification of the haemagglutination-inhibition test used in this laboratory (Burnet, Beveridge, Bull and Clark, 1942). The standard technique is now to use virus which has been concentrated by adsorption to red cells and elution as described by Francis and Salk (1942). The virus is eluted in saline 1/10th the volume of the original allantoic fluid and 33 p.c. of glycerine added. This material may be kept for many months in the refrigerator without appreciable change in its agglutination potency. All specimens of serum from each individual were titrated simultaneously. The titres were converted to a logarithmic scale and the individual increases were totalled for each group and the mean increase calculated. Since the tests were not all done with the same red cells or at the same time, it is not justifiable to compare the means of the titres of the groups, because it has been shown by Hirst (1943) that different titres are often obtained using cells from different fowls or cells which have been held in the refrigerator for varying times. However, a special simultaneous titration was carried out with all the second sera of experiment II A and all the third sera of experiment IV, so that the means of these groups could be compared. A few

sera were also titrated in mice by the usual method and some human sera were titrated by allantoic inoculation of embryos (Burnet and Beveridge, 1943).

## THE EXPERIMENTS.

*Experiment I.*

3·0 ml. of vaccine were inoculated subcutaneously in the right arm of 7 medical student volunteers about 20 years of age and of 2 other adults over 40 years of age; and 2·0 ml. were injected into 2 children 9 and 11 years of age. After an interval of 14 days a second similar injection was administered in the opposite arm. Five of the subjects also received a third injection 15 to 29 days later.

The mean increases after each inoculation, by the haemagglutination-inhibition titrations, are shown in Table 1 together with their standard errors. The main feature of the results was the absence of an appreciable increase after the second inoculation in most persons. Only 2 persons showed an increase of twofold or more with influenza A and only one with influenza B, the others showing only trivial changes. With influenza B the mean increase after the second inoculation is significantly lower than after the first inoculation but the difference between the two increases with influenza A is not statistically significant owing to the considerable variability in the data.

There were 3 individuals who did not show any increase to influenza A after the first inoculation and one who showed none to influenza B: these also failed to show an increase after the second or third inoculation. The other two subjects who were given a third inoculation also showed no appreciable increase after it. The sera of two of the subjects who failed to show an increase after three inoculations were titrated in mice and by allantoic inoculation in eggs. No appreciable increase in antibody was revealed by either method.

That the increase after the second inoculation was not merely delayed was shown by the fact that there was no increase when the five persons given a third inoculation were bled finally, that is 4 to 6 weeks after their second inoculation.

*Experiment II.*

This experiment was planned so that there was a check on any possible change in the potency of the vaccine between the first and second inoculation. Group A, consisting of 13 persons, was given two subcutaneous inoculations of 3·0 ml. of vaccine in different arms at two weeks interval. The same batch of vaccine as used for these two inoculations was also administered to 12 persons in group B at the same time as group A received its second inoculation. Group C was also inoculated at this time with the same material. Group C consisted of 10 persons who had been given 3·0 ml. of a similar type of vaccine 13 months previously and had received an intranasal spray of attenuated influenza viruses A and B 7 months later. As a result of the intranasal spray only two increases in antibody occurred, both with influenza A. These two were eliminated from consideration with influenza A but were retained in the influenza B group. The subjects in all groups were medical students about 20 years of age.

The results of this experiment were even more striking than those in experiment I in that in group A there was not one increase of as much as twofold (i.e. $\times 10^{0\cdot3}$) in titre against either influenza A or B after the second inoculation, whereas after the first inoculation there were 12 increases of this order or greater against each virus. The means of the increases after each inoculation with their standard errors are shown in Table 1. The results obtained with group B showed that the vaccine had lost none of its potency at the time of the second inoculation in group A. The increases after the first inoculations in both groups A and B examined statistically show a significant difference from the quite trivial increases resulting from the second inoculations in group A.

Group C also showed a lower mean increase after the second inoculation than after the first inoculations in any of the 3 groups. A valid comparison cannot be made between the results of the first inoculation of group C and any of the others because a different batch of vaccine was used when the first inoculation was given 13 months previously, but the results of the second inoculation in group C can be legitimately compared with those in the other groups. Statistical examination shows with both influenza A and B a significant difference between the increase after the second inoculation in group C and the increase after the first inoculation in each of the other groups. Therefore, the treatment which group C had received 6 and 13 months previously lessened its response to the vaccine. However, the antibody level in this group was still somewhat elevated as a result of the previous treatment, so although the response to the last inoculation was poor it was sufficient to bring this group to the same final level as the other groups.

The sera of 6 subjects from groups A and C were titrated by allantoic inoculation against strain BEL and the sera of two were titrated against LEE. The results were in close agreement with the haemagglutination-inhibition titrations.

*Experiment III.*

There were available 8 people of varying ages who had had a subcutaneous inoculation of vaccine 2 to 4 months previously. Six had had 3·0 ml. of the usual influenza A and B mixed

but two had had only influenza B. They were all given a second inoculation of 3·0 ml. of the influenza A and B vaccine. The two who had not had the influenza A component before showed an increase of $\times 10^{0\cdot4}$ and $\times 10^{0\cdot9}$: these two were omitted when the mean for the group was calculated, as shown in Table 1. The increase after the second inoculation is significantly lower than after the first inoculations in groups A and B in experiment II but the comparison is not really legitimate since different batches of vaccine were used.

Two subjects in this group showed an appreciable fall in titre against influenza B during the 4 months interval between inoculations and the original titre was not regained after the second inoculation. The actual titres of the sera taken before and after each inoculation were as follows: 15–140–50–65 and 30–140–70–70. All sera were titrated simultaneously.

*Experiment IV.*

This experiment was an attempt to determine whether it was possible by giving a small initial inoculation to produce a refractory state with lower titres than after a dose of the usual size. Eleven students were inoculated with 0·3 ml. of vaccine and two weeks later were given 3·0 ml. in the opposite arm. The mean increases after each inoculation are shown in Table 1.

TABLE 1.

| Experiment. | Inoculum and interval. | No. of subjects. | Mean increases ($Log_{10}$)*. Influenza A. First Inoculation. | Influenza A. Second Inoculation. | Influenza B. First Inoculation. | Influenza B. Second Inoculation. |
|---|---|---|---|---|---|---|
| I | 3·0 ml., 2 weeks | 11 | 0·41 ± ·15 | 0·10 ± ·05 | 0·47 ± ·08 | 0·01 ± ·05 |
| II (A) | 3·0 ml., 2 weeks | 13 | 0·56 ± ·08 | 0·04 ± ·04 | 0·48 ± ·07 | 0·01 ± ·01 |
| (B) | 3·00 ml. | 12 | 0·58 ± ·13 | | 0·84 ± ·15 | |
| (C) | 3·00 ml., 12 months | 10 | 0·77 ± ·07 | 0·18 ± ·05 | 0·64 ± ·07 | 0·17 ± ·05 |
| III | 3·0 ml., 2–4 months | 8 | 0·44 ± ·21 | 0·11 ± ·05 | 0·39 ± ·12 | 0·10 ± ·12 |
| IV | 0·3 and 3·0 ml., 2 weeks | 11 | 0·27 ± ·08 | 0·11 ± ·02 | 0·21 ± ·05 | 0·15 ± ·03 |

* All titres were converted to $log_{10}$ values and the mean increase after each inoculation was calculated in those terms. Thus a mean increase of log 0·3 = mean increase of 2X, and log 0·6 = 4X.

The mean total increase in experiment IV is less than the increase after an initial dose of 3·0 ml. in any of the other experiments though in most instances the difference is not statistically significant. No definite conclusion is justified since different batches of vaccine were used in the different experiments, but the results are at least suggestive. The results of this experiment have been compared in another way with the results obtained in experiment II, group A. All the second sera from experiment II, group A, and all the third sera from experiment IV were titrated simultaneously. The mean final titres of the second sera from experiment II A expressed as $log_{10}$ were 2·10 ± ·11 with influenza A and 2·08 ± ·06 with influenza B, compared with 1·82 ± ·03 and 1·86 ± ·07 for the third sera in experiment IV.

## DISCUSSION.

The experiments have demonstrated that most persons, after receiving a subcutaneous inoculation of 3·0 ml. of the influenza vaccine described, do not show a significant increase in antibody as a result of a second similar inoculation given two weeks after the first and that some degree of refractoriness to a second inoculation persists for six months or a year. It should be noted that these results were obtained with normal adults nearly all of whom were known to have a basic immunity to influenza so that the first subcutaneous inoculation was not a primary antigenic stimulus. Different results might well be obtained in young children who had never had influenza.

It has been demonstrated by several investigators that the increase in antibody after subcutaneous inoculation of influenza virus varies greatly from person to

person. The proportion of persons who fail to show an increase is greatest among those with the high initial antibody levels, but there is by no means an absolute correlation between height of initial antibody and failure to show an increase (Horsfall, Lennette and Rickard, 1941; Hirst, Rickard, Whitman and Horsfall, 1942; Francis, Pearson, Sullivan and Brown, 1943). This has also been our experience. The question arises whether the refractory state of subjects after one subcutaneous inoculation is entirely explained by the fact that most of their titres have been raised to levels which, when occurring naturally, are frequently associated with refractoriness. Examination of our detailed protocols suggests that vaccinated persons are more refractory than are the few unvaccinated persons with similarly high titres occurring naturally. However, to provide a definite answer to this question would require an investigation involving a very large number of persons, owing to the great variability between individuals even with the same antibody level. Some further evidence on this point is provided by the results of experiment IV. These suggest that by giving a small initial inoculation it may be possible to produce a refractory state with lower titres than when a large initial dose is given, although the results of this experiment were not at all conclusive. Also in experiment III two subjects whose titre had fallen four months after the first inoculation, when given a second inoculation failed to regain the titre reached after the first inoculation. It is also relevant that Hirst *et al.* (1942) showed that by inoculating relatively massive doses, equivalent to 34 ml. of allantoic fluid, it is possible to obtain a mean increase of $\times 10^{1 \cdot 03}$, which is considerably greater than he obtained with inocula about the same size as we used in the present experiments, or than we have obtained. This indicates that the level of the titres after our first inoculations were probably not the maximal titres which could be attained, but nevertheless the subjects were then refractory to a second inoculation. Therefore, it seems likely that the size of the initial inoculum largely determines the level of the titre during the refractory period and that refractoriness is not due to the antibody having reached the maximum level which the person is capable of obtaining.

It is well known that during influenza epidemics many cases of symptomatic infection occur which do not show an increase in antibody against influenza virus and yet they are indistinguishable clinically from the cases that do show an increase. In an epidemic studied by Horsfall, Lennette, Rickard and Hirst (1941) of 365 cases of influenza in unvaccinated persons, 66 (18 p.c.) were serologically negative, while in 193 cases in vaccinated persons, 56 (29 p.c.) were serologically negative. Siegal, Muckenfuss, Schaeffer, Wilcox and Leider (1942) showed that there is a higher percentage of serologically negative cases in persons with a high initial titre than in others. They also reported isolation of influenza virus from one serologically negative case and quote three similar instances from the literature. At least many of the serologically negative cases occurring during an epidemic of influenza A must be presumed to be cases of influenza A, but it has been difficult to understand that there was no antibody increase produced as a result of these acute febrile illnesses. The results reported in this paper throw some light on this problem, especially in those cases which occurred with greater frequency in the vaccinated groups. The vaccination had "protected" against an increase in antibody rather than against the disease.

Siegal *et al.* (1942) inoculated subjects at yearly intervals for three successive years with vaccine of apparently the same potency. During the second and third year the antibody was not raised to as high a level as during the first year. When a more potent vaccine was used during the fourth year the antibody titre rose to about the same titre as after vaccination in the first year. The authors do not offer any explanation of this observation but it is likely that it illustrates the same phenomenon as reported in this paper.

SUMMARY.

Persons who have had one subcutaneous injection of formolized influenza virus mostly fail to get an increase in antibody as a result of a second similar injection given two weeks later. Even six months or perhaps a year after the first injection the increase following a second injection is less than in persons never previously vaccinated.

*Acknowledgments.* Mr. E. A. Cornish of the Council for Scientific and Industrial Research very kindly carried out the statistical examination of the data. It is a pleasure also to acknowledge the help and collaboration of Miss Joyce D. Stone with several of the experiments and helpful advice from Dr. F. M. Burnet.

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# SUPPRESSION OF ANTIGENICITY OF INFLUENZA VIRUS BY ADMIXTURE WITH HOMOLOGOUS ANTISERUM

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*(Accepted for publication 2nd October, 1944.)*

Second injections of influenza virus in man usually do not give rise to a significant increase in antibody, and there is evidence suggesting that this refractory state is not due to the antibody having reached the maximum level which the person is capable of obtaining (Beveridge, 1944). A possible explanation of the refractory state is that the virus injected is rendered non-antigenic by contact with the antibody formed as a result of the first injection. Experiments were, therefore, conducted in rabbits and mice to determine whether admixture with homologous antiserum did suppress the antigenicity of influenza virus.

The influenza A strain BEL was used with the rabbits and strain "Melbourne" was used in the mice. The techniques employed were the same as in the preceding paper (Beveridge, 1944).

## THE EXPERIMENTS.

*Experiment I.*

Rabbit antiserum was available which had been prepared about a year previously by multiple intravenous injections of influenza strain BEL. This was titrated against BEL allantoic fluid virus by allantoic inoculation of 11 day embryos and it was found that undiluted serum neutralized equal parts of undiluted virus but serum diluted 1/10 did not give neutralization. The virus alone infected eggs by allantoic inoculation of 0·05 ml. of a dilution of $10^{-4}$. Formalin was then added to the virus in a concentration of 0·025 p.c.

Two rabbits were inoculated intravenously with a mixture of 1 ml. antiserum and 1 ml. formolized virus, and two with a mixture of 1 ml. saline and 1 ml. formolized virus. The mixtures were held at 37° C. for 30 minutes before inoculation. Two weeks later fresh mixtures of the same materials were made and the inoculations repeated. Final bleedings were made two weeks after the second inoculations. The sera were titrated by haemagglutination-inhibition and by allantoic inoculation and the results are shown in Table 1.

TABLE 1.

*Suppression of antigenicity.*

| Experiment. | Rabbit. | Inoculum. | Haemagglutination-inhibition. | | | Allantoic inoculation. | | |
|---|---|---|---|---|---|---|---|---|
| | | | SI | SII | SIII | SI | SII | SIII |
| I | 18 | Antiserum and virus | (130) | (120) | (120) | 0 | – | 0 |
| | 19 | " " " | (130) | (120) | (120) | 0 | - | 0 |
| | 20 | Saline and virus | (270) | (270) | *620* | 0 | 6 | 7 |
| | 21 | " " " | (90) | (100) | *600* | 0 | 0 | 7 |
| III | 25 | Antiserum and virus | (350) | (300) | (300) | 0 | – | 0 |
| | 26 | " " " | (160) | (150) | (160) | 0 | – | 0 |
| | 27 | " " " | (160) | (160) | (200) | 0 | – | 0 |
| | 28 | Normal serum and virus | (180) | *360* | *800* | 0 | 2·5 | 7 |
| | 29 | " " " " | (140) | *320* | *800* | 0 | 3 | 7 |
| | 30 | " " " " | (120) | *440* | *1,200* | 0 | 5 | 7 |

The titres of haemagglutination-inhibition are the actual dilutions at the end points; the titres in parentheses are due to non-specific inhibition which is always present to a considerable dilution in normal rabbit sera. The titres of the allantoic inoculations are the $\log_{10}$ of the number of infective doses of virus neutralized by an equal volume of the serum concerned. SI, SII, SIII signify respectively the sera taken before the first inoculation, before the second inoculation and two weeks after the second inoculation.

Haemagglutination-inhibition titrations with rabbit sera have the disadvantage that there is always a high non-specific titre. The two rabbits receiving the antiserum-virus mixture showed the same titre in sera taken before inoculation and in the final sera, whereas the two receiving virus and saline showed an increase over their initial non-specific titre. By allantoic inoculation the initial sera and the final sera from the two rabbits receiving antiserum-virus mixture showed no trace of antibody whereas the final sera from the rabbits given virus plus saline each neutralized $10^7$ infective doses. Thus there was no antibody produced in the rabbits inoculated with the virus antiserum mixture although those given virus and saline reached a high titre. The two rabbits with no antibody were subsequently given two intravenous inoculations of the virus and saline mixture after which both developed a haemagglutination-inhibition titre of 350.

*Experiment II.*

Two rabbits (R22 and R23) were given, at fortnightly intervals, a series of 4 injections each of 1 ml. of a 1: 100 dilution in saline of the same formolized virus as used in experiment I. The sera taken just before the fourth injection showed allantoic titres of 7 for R22 and 4 for R23, that is, the serum of R22 neutralized $10^7$ infective doses of virus and the serum of R23 neutralized $10^4$ doses. When bled again 8 weeks later R22 still had a titre of 5.

*Experiment III.*

This experiment was carried out along the same lines as experiment I using 3 rabbits in each group. The antiserum used in this experiment was obtained from one of the rabbits given virus and saline in experiment I. The virus used was grown for 24 hours instead of the usual 42 and had a titre of $10^8$ infective doses per 0·05 ml. for embryos inoculated in the allantoic cavity. A mixture of equal parts undiluted virus and antiserum gave a nearly neutral mixture; when inoculated into the allantoic cavity only one out of 6 eggs became infected. The virus was formolized and 3 rabbits in group I were inoculated intravenously with 2 ml. of a mixture of equal parts of virus and antiserum, and 3 rabbits in group II were inoculated with 2 ml. of a mixture of virus and normal rabbit serum. The mixtures were held in the refrigerator for one hour before inoculation.

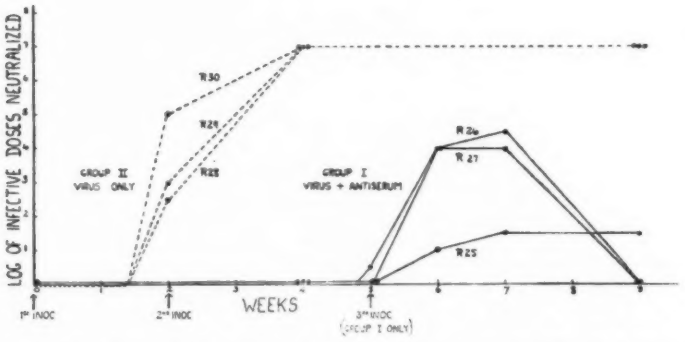


**Fig. 1.** Antibody titrations (allantoic method) in rabbits given two inoculations of formolized influenza virus (group II) or three inoculations of the same material mixed with antiserum (group I).

Inoculations were repeated in all rabbits after an interval of two weeks and, after a further interval of three weeks, group I was re-inoculated but not group II. All rabbits were bled before the first inoculation and after 2, 4 and 9 weeks; in addition those in group I were bled after 5, 6 and 7 weeks.

The sera were titrated by haemagglutination-inhibition and by allantoic inoculation. The results obtained with the first three bleedings are shown in Table 1, and the allantoic titrations over the whole period are shown graphically in Fig. 1. The rabbits receiving virus and normal serum showed a moderate amount of antibody 2 weeks after the first dose, and 2 weeks after the second dose a high level was reached which was maintained until the 9th week. The rabbits receiving the virus and antiserum had no detectable antibody 4 weeks after the first inoculation and only one had a trace by the 5th week, i.e. at the time of the third inoculation. At the 6th and 7th weeks moderate levels were reached but these were not maintained at the 9th week.

*Experiment IV.*

This experiment was designed to determine whether admixture with human antiserum would inhibit the immunizing power of the formolized virus in mice. The mouse-adapted "Melbourne" strain was used in the form of allantoic fluid virus which had an allantoic titre of $10^7$. Serum from a subject who had a high titre after subcutaneous inoculation with BEL was chosen. A mixture of undiluted serum and undiluted virus gave a nearly neutral mixture; when inoculated into the allantoic cavity of 4 eggs, none showed virus after 2 days incubation but all four did after 3 days. Groups of 8 mice were inoculated intraperitoneally twice, at an interval of 2 weeks. Group I received 0·05 ml. of formolized virus, group II 0·05 ml. formolized virus mixed with 0·05 ml. normal horse serum, group III 0·05 ml. formolized virus mixed with 0·05 ml. of the human serum, group IV were unvaccinated controls. Two weeks after the second inoculation all mice were given a challenge dose of 0·05 ml. of 1: 100 dilution of active virus intranasally.

The mice in groups I and II showed almost complete resistance; only 3 in each group showed moderate lesions when killed a week after the challenge dose, the others being normal. The mice in group III which received formolized virus mixed with the human serum, showed no appreciable immunity: 6 of the 8 were dead by the 7th day and the other two showed extensive lesions. All the unvaccinated controls were dead by the 7th day. Thus admixture of the formolized virus with the human serum deprived it of its immunizing power.

## DISCUSSION.

The experiments demonstrate that in rabbits and mice homologous antibody to a large extent suppresses the antigenicity of formolized influenza virus A. The antibody response following three inoculations of antiserum and virus in experiment III was somewhat less than after three inoculations of 1/100 dilution of virus in experiment II and was less sustained.

It is well known that fully neutralized or over-neutralized mixtures of diphtheria or tetanus toxin or toxoid and homologous antiserum are only poorly antigenic compared with underneutralized mixtures (Glenny and Sudmersen, 1921; Ramon and Laffaille, 1925; Hartly, 1926), and that bacteria treated with antibody are poorer antigens than when not so treated (Armstrong, 1925). The suppression of antigenicity demonstrated in our experiments is, of course, a different principle from the one involved in attempts at immunization by inoculation of relatively small amounts of active virus mixed with antiserum. Such mixtures, when neutral, usually produce no immunity, but this is because the virus is rendered non-infective and incapable of producing an adequate mass of antigen by its multiplication.

Probably the failure to get an antibody response in man to a second inoculation of influenza virus is due to the antigen inoculated being rendered ineffective by union with the antibody produced as a result of the first inoculation. The same phenomenon, i.e., suppression of antigenicity by the circulating antibody, may also account for the failure of some persons to get an increase of antibody after a single subcutaneous injection or after a natural attack of the disease.

## SUMMARY.

Treatment of formolized influenza virus A with an appropriate amount of homologous antiserum largely suppresses its capacity to produce antibody in rabbits or immunity in mice.

Suppression of antigenicity by antibody *in vivo* is the probable explanation of the failure of vaccinated persons to respond to second inoculations of influenza virus.

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